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(54) Title: ANTISENSE IAP NUCLEOBASE OLIGOMERS AND USES THEREOF

(57) Abstract: The present invention features nucleobase oligomers that hybridize to IAP polynucleotides, and methods for using them to enhance apoptosis and treat proliferative diseases.



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## ANTISENSE IAP NUCLEOBASE OLIGOMERS AND USES THEREOF

### Background of the Invention

The invention relates to antisense IAP nucleobase oligomers and methods of using them to induce apoptosis.

One way by which cells die is referred to as apoptosis, or programmed cell death. Apoptosis often occurs as a normal part of the development and maintenance of healthy tissues. The process may occur so rapidly that it is difficult to detect.

The apoptosis pathway is now known to play a critical role in embryonic development, viral pathogenesis, cancer, autoimmune disorders, and neurodegenerative diseases, as well as other events. The failure of an apoptotic response has been implicated in the development of cancer, autoimmune disorders, such as lupus erythematosus and multiple sclerosis, and in viral infections, including those associated with herpes virus, poxvirus, and adenovirus.

The importance of apoptosis in cancer has become clear in recent years. The identification of growth promoting oncogenes in the late 1970's gave rise to an almost universal focus on cellular proliferation that dominated research in cancer biology for many years. Long-standing dogma held that anti-cancer therapies preferentially targeted rapidly dividing cancer cells relative to "normal" cells. This explanation was not entirely satisfactory, since some slow growing tumors are easily treated, while many rapidly dividing tumor types are extremely resistant to anti-cancer therapies. Progress in the

cancer field has now led to a new paradigm in cancer biology wherein neoplasia is viewed as a failure to execute normal pathways of programmed cell death. Normal cells receive continuous feedback from their neighbors through various growth factors, and commit “suicide” if removed from this context. Cancer cells somehow bypass these commands and continue inappropriate proliferation. It is now believed that many cancer therapies, including radiation and many chemotherapies, previously thought to act by causing cellular injury, actually work by triggering apoptosis.

Both normal cell types and cancer cell types display a wide range of susceptibility to apoptotic triggers, although the determinants of this resistance are only now under investigation. Many normal cell types undergo temporary growth arrest in response to a sub-lethal dose of radiation or cytotoxic chemical, while cancer cells in the vicinity undergo apoptosis. This differential effect at a given dose provides the crucial treatment window that allows successful anti-cancer therapy. It is therefore not surprising that resistance of tumor cells to apoptosis is emerging as a major category of cancer treatment failure.

Several potent endogenous proteins that inhibit apoptosis have been identified, including Bcl-2, and IAP (inhibitor-of apoptosis) families in mammalian cells. Certain members of the latter family directly inhibit terminal effector caspases, i.e. casp-3 and casp-7, engaged in the execution of cell death, as well as the key mitochondrial initiator caspase, casp-9, important to the mediation of cancer chemotherapy induced cell death. The IAPs are the only known endogenous caspase inhibitors, and thus play a central role in the regulation of apoptosis.

The IAPs have been postulated to contribute to the development of some cancers, and a postulated causal chromosomal translocation involving one particular IAP (cIAP2/HIAP1) has been identified in MALT lymphoma. A recent correlation between elevated XIAP, poor prognosis, and short survival has been demonstrated in patients with

acute myelogenous leukemia. XIAP was highly over-expressed in many tumor cell lines of the NCI panel.

There exists a need for improved cancer therapeutics and, in particular, therapeutics that can induce cancer cells to undergo apoptosis and override anti-apoptotic signals provided in such cells.

### **Summary of the Invention**

In general, the invention features methods and reagents useful for inducing apoptosis in a cell. The methods and reagents of the invention are useful in treating cancers, and other proliferative diseases.

The present invention features nucleobase oligomers, particularly oligonucleotides, for use in modulating the function of a polynucleotide encoding an IAP. These oligomers reduce the amount of an IAP produced, allowing a cell normally expressing the IAP to undergo apoptosis. This is accomplished by providing nucleobase oligomers that specifically hybridize with one or more polynucleotides encoding an IAP. The specific hybridization of the nucleobase oligomer with an IAP polynucleotide (e.g., RNA, DNA) interferes with the normal function of that IAP polynucleotide, reducing the amount of IAP protein produced. This modulation of function of a target nucleic acid by compounds that specifically hybridize to the target is generally referred to as "antisense."

In one aspect, the invention features a nucleobase oligomer of up to 30 nucleobases in length, the oligomer including at least eight consecutive nucleobases of a sequence selected from SEQ ID NOs: 1-99, 143, 147, 151, 163-260, 287, 289, and 300-460. Desirably, when administered to a cell, the oligomer inhibits expression of an IAP.

In certain embodiments, the nucleobase oligomer includes a sequence selected from SEQ ID NOs: 1-99, 143, 147, 151, 163-260, 287, 289, and 300-460. It is desirable that the nucleobase oligomer consists of (or essentially of) one or more of the foregoing



SEQ ID NOs. For example, the nucleobase oligomer may be a XIAP antisense nucleic acid that includes a sequence chosen from SEQ ID NOs 97, 98, 99, 143, 147, 151, 287, and 289, a HIAP1 antisense nucleic acid that includes a sequence chosen from SEQ ID NOs 300-389, or a HIAP2 antisense nucleic acid includes a sequence chosen from SEQ ID NOs 390-460. In a particularly desirable embodiment, the invention features a nucleobase oligomer having eleven DNA residues flanked on each side by four 2'-O-methyl RNA residues, and consists of one of the following sequences: 5'-AUUGGTTTCAATGTGUUCU-3' (SEQ ID NO: 155); 5'-ACACGACCGCTAAGAAACA-3' (SEQ ID NO: 16); 5'-ACAGGACTACCACTTGGAA-3' (SEQ ID NO: 157); 5'-UGCCAGTTTGATGCUGAA-3' (SEQ ID NO: 27); 5'-GCUGAGTCTCCATATUGCC-3' (SEQ ID NO: 141); 5'-UCGGGTATATGGTGTCTUGA-3' (SEQ ID NO: 41); 5'-AAGCACTGCACTTGUCAC-3' (SEQ ID NO: 47); 5'-CCGGCCCAAACAAAGAAG-3' (SEQ ID NO: 51); 5'-ACCCTGGATACCATTUAGC-3' (SEQ ID NO: 63); 5'-UGUCAGTACATGTTGGCUC-3' (SEQ ID NO: 161); and 5'-UGCACCCTGGATACCAUUU-3' (SEQ ID NO: 151).

A nucleobase oligomer of the present invention may include at least one modified linkage (e.g., a phosphorothioate, a methylphosphonate, a phosphotriester, a phosphorodithioate, or a phosphoselenate linkage), modified nucleobase (e.g., a 5-methyl cytosine), and/or a modified sugar moiety (e.g., a 2'-O-methoxyethyl group or a 2'-O-methyl group). In one embodiment, the oligomer is a chimeric oligomer (e.g., an oligonucleotide that includes DNA residues linked together by phosphorothioate or phosphodiester linkages, flanked on each side by at least one, two, three, or four 2'-O-methyl RNA residue linked together by a phosphorothioate linkage).

In another aspect, the invention features a method of enhancing apoptosis in a cell. This method includes the step of administering to the cell a nucleobase oligomer of the present invention so that expression of an IAP (e.g., XIAP, HIAP1, or HIAP2) is

inhibited. The nucleobase oligomer may be, e.g., a component of an antisense compound, a double-stranded RNA, or a ribozyme. This administering step may be performed alone, or in combination with a second step (e.g., administration of a chemotherapeutic agent, a biological response modifying agent, and/or a chemosensitizer). The cell can be *in vitro* or *in vivo*. In one embodiment, the cell is a cancer cell (e.g., a human cancer cell) or a cell of lymphoid or myeloid origin.

In a related aspect, the invention features a method for treating an animal (e.g., a human) having a proliferative disease (e.g., a cancer, lymphoproliferative disorder, or myelodysplastic syndrome) or preventing the development of such a disease, by administering to the animal an effective amount of a nucleobase oligomer of the present invention.

The cancer may be, for example, acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, myelodysplastic syndrome, chronic lymphocytic leukemia, polycythemia vera, lymphoma, Hodgkin's disease, Waldenstrom's macroglobulinemia, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder

carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, schwannoma, meningioma, melanoma, neuroblastoma, or retinoblastoma. When treating a cancer, it may be desirable to also administer one or more chemotherapeutic agents, biological response modifying agents, and/or chemosensitizers. Desirably, the administration of one or more of these agents is within five days of the administration of the nucleobase oligomer. Exemplary chemotherapeutic agents are adriamycin (doxorubicin), vinorelbine, etoposide, taxol, and cisplatin. While any route of administration that results in an effective amount at the desired site may be used, particularly desirable routes are by intravenous and intratumoral administration.

In another aspect, the invention features a pharmaceutical composition that includes a nucleobase oligomer of the present invention and a pharmaceutically acceptable carrier. If desirable, the pharmaceutical composition may further include additional components (e.g., a colloidal dispersion system or a chemotherapeutic agent).

The invention also features a catalytic RNA molecule capable of cleaving XIAP, HIAP1, or HIAP2 mRNA. In desirable embodiments, the catalytic RNA molecule includes, in its binding arms, at least eight consecutive nucleobases corresponding to a nucleobase oligomer of the invention (e.g., a nucleobase sequence of any one of Tables 1, 2, 6, and 7). The RNA molecule is desirably in a hammerhead motif, but may also be in a hairpin, hepatitis delta virus, group 1 intron, VS RNA or RNaseP RNA motif.

The invention also features an expression vector including a nucleic acid encoding one or more catalytic RNA molecules of the invention positioned for expression in a mammalian cell.

The invention also features a method of treating an animal having a cancer or lymphoproliferative disorder by administering to the animal an effective amount of a

catalytic RNA molecule described above, or an expression vector encoding such a catalytic RNA molecule.

In still another aspect, the invention features a double-stranded RNA molecule having between 21 and 29 nucleobases, wherein at least eight consecutive nucleobases corresponding to a sequence of any one of Tables 1, 2, 6, and 7 are present.

In a related aspect, the invention also features a double-stranded RNA molecule having between 50 and 70 nucleobases, the RNA molecule having a first domain of between 21 and 29 nucleobases that include least eight consecutive nucleobases corresponding to a sequence of any one of Tables 1, 2, 6, and 7; a second domain complementary to the first domain, and a loop domain situated between the first and second domains such that the first and second domains are capable of duplexing to form a double-stranded RNA molecule. The invention also features an expression vector (e.g., an adenoviral vector or a retroviral vector) encoding such a double stranded RNA.

The invention also features a method of treating an animal having a cancer or lymphoproliferative disorder by administering to the animal an effective amount of a double-stranded RNA molecule described above

By a "nucleobase oligomer" is meant a compound that includes a chain of at least eight nucleobases joined together by linkage groups. Included in this definition are natural and non-natural oligonucleotides, both modified and unmodified, as well as oligonucleotide mimetics such as Protein Nucleic Acids, locked nucleic acids, and arabinonucleic acids. Numerous nucleobases and linkage groups may be employed in the nucleobase oligomers of the invention, including those described in detail herein in the section entitled "Oligonucleotides and other nucleobase oligomers," *infra*.

"Protein" or "polypeptide" or "polypeptide fragment" means any chain of more than two amino acids, regardless of post-translational modification (e.g., glycosylation or

phosphorylation), constituting all or part of a naturally occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

“Apoptosis” means the process of cell death wherein a dying cell displays a set of well-characterized biochemical hallmarks that include cell membrane blebbing, cell soma shrinkage, chromatin condensation, and DNA laddering. Cells that die by apoptosis include neurons (e.g., during the course of neurodegenerative diseases such as stroke, Parkinson’s disease, and Alzheimer’s disease), cardiomyocytes (e.g., after myocardial infarction or over the course of congestive heart failure), and cancer cells (e.g., after exposure to radiation or chemotherapeutic agents). Environmental stress (e.g., hypoxic stress) that is not alleviated may cause a cell to enter the early phase of the apoptotic pathway, which is reversible (i.e., cells at the early stage of the apoptotic pathway can be rescued). At a later phase of apoptosis (the commitment phase), cells cannot be rescued, and, as a result, are committed to die.

Proteins and compounds known to stimulate and inhibit apoptosis in a diverse variety of cells are well known in the art. For example, intracellular expression and activation of the caspase (ICE) family induces or stimulates apoptotic cell death, whereas expression of the IAPs or some members of the Bcl-2 family inhibit apoptotic cell death. In addition, there are survival factors that inhibit cell death in specific cell types. For example, neurotrophic factors, such as NGF inhibit neuronal apoptosis.

By “IAP gene” is meant a gene encoding a polypeptide having at least one BIR domain and that is capable of modulating (inhibiting or enhancing) apoptosis in a cell or tissue when provided by other intracellular or extracellular delivery methods (see, e.g., U.S. Patent No. 5,919,912). In preferred embodiments, the IAP gene is a gene having about 50% or greater nucleotide sequence identity (e.g., at least 85%, 90%, or 95%) to at least one of human or murine XIAP, HIAP1, or HIAP2 (each of which is described in U.S. Patent No. 6,156,535). Preferably the region of sequence over which identity is

measured is a region encoding at least one BIR domain and a ring zinc finger domain. Mammalian IAP genes include nucleotide sequences isolated from any mammalian source. Preferably the mammal is a human.

By "IAP protein" or "IAP polypeptide" is meant a polypeptide, or fragment thereof, encoded by an IAP gene.

By "IAP biological activity" is meant any activity known to be caused *in vivo* or *in vitro* by an IAP polypeptide.

By "enhancing apoptosis" is meant increasing the number of cells that apoptose in a given cell population (e.g., cancer cells, lymphocytes, fibroblasts, or any other cells). It will be appreciated that the degree of apoptosis enhancement provided by an apoptosis-enhancing compound in a given assay will vary, but that one skilled in the art can determine the statistically significant change in the level of apoptosis that identifies a nucleobase oligomer that enhances apoptosis otherwise limited by an IAP. Preferably, "enhancing apoptosis" means that the increase in the number of cells undergoing apoptosis is at least 10%, more preferably the increase is 25% or even 50%; and most preferably the increase is at least one-fold, relative to cells not administered a nucleobase oligomer of the invention but otherwise treated in a substantially similar manner. Preferably the sample monitored is a sample of cells that normally undergo insufficient apoptosis (i.e., cancer cells). Methods for detecting changes in the level of apoptosis (i.e., enhancement or reduction) are described herein.

By a nucleobase oligomer that "inhibits the expression" of a target gene (e.g., an IAP) is meant one that reduces the amount of a target mRNA, or protein encoded by such mRNA, by at least about 5%, more desirable by at least about 10%, 25%, or even 50%, relative to an untreated control. Methods for measuring both mRNA and protein levels are well-known in the art; exemplary methods are described herein.

“Hybridization” means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleobases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

By “proliferative disease” is meant a disease that is caused by or results in inappropriately high levels of cell division, inappropriately low levels of apoptosis, or both. For example, cancer is an example of a proliferative disease. Examples of cancers include, without limitation, leukemias (e.g., acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin’s disease, non-Hodgkin’s disease), Waldenstrom’s macroglobulinemia, heavy chain disease, and solid tumors such as sarcomas and carcinomas (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing’s tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm’s tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, schwannoma, meningioma, melanoma, neuroblastoma, and

retinoblastoma). Lymphoproliferative disorders are also considered to be proliferative diseases.

Preferably, a nucleobase oligomer of the invention is capable of enhancing apoptosis and/or decreasing IAP mRNA or protein levels when present in a cell that normally does not undergo sufficient apoptosis. Preferably the increase is by at least 10%, relative to a control, more preferably 25%, and most preferably 1-fold or more. Preferably a nucleobase oligomer of the invention includes from about 8 to 30 nucleobases, wherein at least eight consecutive nucleobases are from a sequence selected from SEQ ID NOs: 1-99, 143, 147, 151, 163-260, 287, 289, and 300-460. A nucleobase oligomer of the invention may also contain, e.g., an additional 20, 40, 60, 85, 120, or more consecutive nucleobases that are complementary to an IAP polynucleotide. The nucleobase oligomer (or a portion thereof) may contain a modified backbone. Phosphorothioate, phosphorodithioate, and other modified backbones are known in the art. The nucleobase oligomer may also contain one or more non-natural linkages.

By "chemotherapeutic agent" is meant an agent that is used to kill cancer cells or to slow their growth. Accordingly, both cytotoxic and cytostatic agents are considered to be chemotherapeutic agents.

By "biological response modifying agent" is meant an agent that stimulates or restores the ability of the immune system to fight disease. Some, but not all, biological response modifying agents may slow the growth of cancer cells and thus are also considered to be chemotherapeutic agents." Examples of biological response modifying agents are interferons (alpha, beta, gamma), interleukin-2, rituximab, and trastuzumab.

By "chemosensitizer" is meant an agent that makes tumor cells more sensitive to the effects of chemotherapy.

By "an effective amount" is meant the amount of a compound (e.g., a nucleobase oligomer) required to ameliorate the symptoms of a disease, inhibit the growth of the



target cells, reduce the size or number of tumors, inhibit the expression of an IAP, or enhance apoptosis of target cells, relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of abnormal proliferation (i.e., cancer) varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an “effective” amount.

By “lymphoproliferative disorder” is meant a disorder in which there is abnormal proliferation of cells of the lymphatic system (e.g., T-cells and B-cells), and includes multiple sclerosis, Crohn’s disease, lupus erythematosus, rheumatoid arthritis, and osteoarthritis.

By “ribozyme” is meant an RNA that has enzymatic activity, possessing site specificity and cleavage capability for a target RNA molecule. Ribozymes can be used to decrease expression of a polypeptide. Methods for using ribozymes to decrease polypeptide expression are described, for example, by Turner et al., (Adv. Exp. Med. Biol. 465:303-318, 2000) and Norris et al., (Adv. Exp. Med. Biol. 465:293-301, 2000).

By “reporter gene” is meant a gene encoding a polypeptide whose expression may be assayed; such polypeptides include, without limitation, glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and beta-galactosidase.

By “promoter” is meant a polynucleotide sufficient to direct transcription.

By “operably linked” is meant that a first polynucleotide is positioned adjacent to a second polynucleotide that directs transcription of the first polynucleotide when appropriate molecules (e.g., transcriptional activator proteins) are bound to the second polynucleotide.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

### Brief Description of the Drawings

Figs. 1A-1L are graphs showing the effect of antisense XIAP oligonucleotides on XIAP protein expression, relative to total protein (Figs. 1A, 1C, 1E, 1G, 1I, and 1K). Figs. 1B, 1D, 1F, 1H, 1J, and 1L are the total protein concentration values for each oligonucleotide transfection compared to mock transfection results that were used to normalize the above XIAP protein results.

Figs. 2A-2C are graphs showing the effects of various antisense XIAP oligonucleotides, alone or in combination, on XIAP RNA (Fig. 2A) and protein (Fig. 2B). Fig. 2C is a graph of the total protein concentration values for each oligonucleotide transfection compared to mock transfection results, which were used to normalize the XIAP protein results shown in Fig. 2B.

Figs. 3 and 4 are graphs showing the effects of 4X4 mixed backbone (MBO) FG8 or E12 oligonucleotides in amounts of 31 nM (Fig. 3) or 63 nM (Fig. 4). H460 lung carcinoma cells were transfected for 18 hours on one, two, or three consecutive days using 125 nM MBOs and Lipofectamine 2000. Samples for western analysis were harvested at the indicated time. Scanning densitometry was performed, and XIAP protein levels were normalized to GAPDH and compared to a mock control set to 100%. The indicated percentages express % XIAP protein knockdown versus specific scrambled controls.

Figs. 5A-5D are graphs of the effects of antisense XIAP oligonucleotides on cell viability (Figs. 5A, 5C, and 5D), and chemosensitization in the presence of adriamycin (Fig. 5B).

Fig. 6 is a graph showing oligonucleotide-mediated specific down-regulation of XIAP mRNA in H460 cells *in vitro*. Depicted are XIAP mRNA levels in H460 cells treated with Lipofectamine 2000 alone (LFA) or Lipofectamine 2000 with 1.2  $\mu$ M of

oligonucleotides F3, G4, C5, AB6, DE4 or D7, or a respective reverse polarity (RP) or scrambled (SC) oligonucleotide control. Real-time RT-PCR quantification of the relative amount of XIAP mRNA was performed at 6 hours of transfection. All data are presented as the mean  $\pm$  standard deviation (SD) of triplicates from a representative experiment. The level of XIAP mRNA in untreated cells (control) maintained under identical experimental conditions was assigned a value of 1.

Fig. 7 is a graph showing XIAP RNA levels in H460 cells after transfection with various PS-XIAP oligonucleotides. H460 human lung cancer cells were transfected for 6 hours using 1  $\mu$ M PS-oligonucleotides and Lipofectamine 2000. Cells were then harvested for Taqman analysis.

Fig. 8 is a graph showing XIAP RNA levels in H460 cells 9 hours post-transfection with 4X4 MBOs. H460 cells were transfected for 9 hours using 4X4 MBOs at 62.5 nM to 1  $\mu$ M and Lipofectamine 2000. The cells were then harvested for Taqman analysis.

Fig. 9 is a graph showing XIAP protein knockdown in H460 cells 24 hours after transfection with 4X4 MBOs. H460 cells were transfected for 24 hours using 1  $\mu$ M 4X4 MBOs at 1  $\mu$ M and Lipofectamine 2000. The cells were then harvested for western blot analysis. Scanning densitometry was performed, and XIAP protein levels were normalized to actin and compared to their specific scrambled (sm, rm) controls, which were set at 100%.

Figs. 10A and 10B are schematic illustrations showing antisense-mediated specific downregulation of XIAP protein in H460 cells *in vitro*. Depicted are XIAP protein levels in H460 cells treated with Lipofectamine 2000 alone (LFA) or LFA plus 1.2  $\mu$ M of XIAP oligonucleotides F3, G4, or C5, or their respective oligonucleotide controls (RP, SC). XIAP protein levels were analyzed by western blotting (Fig. 10A), and the amount of

protein was quantified by densitometry (Fig. 10B). XIAP levels were normalized to cellular actin levels and compared to untreated control (CNT) levels.

Figs. 11A and 11B are schematic illustrations showing XIAP oligonucleotide-mediated effects on caspase activation. The effect of XIAP oligonucleotides F3, G4, or C5, or their respective RP and SC ODN controls at 1.2  $\mu$ M on the expression of pro-caspase-3, PARP (both full length (116 kDa) and processed (85 kDa)) (Fig. 10A) and Bcl-2 and Bax protein levels (Fig. 10B) in transfected H460 cells compared to control is shown. Proteins expression was analyzed by western blotting. Bcl-2 and Bax protein levels were normalized to cellular actin levels and quantified by densitometry. The ratio of Bcl-2/Bax is presented as the mean of two or three independent experiments, and the ratio in control (CNT) cells set at 1.

Figs. 12A and 12B are schematic illustrations showing XIAP oligonucleotide-specific induction of apoptosis. Induction of apoptosis was measured in H460 cells treated with 1.2  $\mu$ M of XIAP G4 AS oligonucleotide, G4 SC oligonucleotide or untreated control (CNT). Fig. 12A shows the percentage of cells having sub-G0/G1 (apoptotic) DNA content, as measured by propidium iodide (PI) staining and flow cytometry. Fig. 12B shows nuclear morphology of oligonucleotide-treated H460 cells stained with DAPI. Arrows indicate cells that have characteristic apoptotic morphology of nuclear DNA condensation or fragmentation.

Fig. 13A is a graph showing the effect of XIAP G4 AS oligonucleotide treatment on the viability of H460 cells. Cells were treated with an increasing concentration of LFA alone or LFA-oligonucleotide complexes with G4 AS oligonucleotides or G4 SC oligonucleotides, and cells viability was determined by MTT assay after 24 hours of treatment. The data represent the mean  $\pm$  SD of three independent experiments.

Fig. 13B is a graph showing the percentage of dead H460 cells after treatment with LFA and complexes with G4 AS oligonucleotides or G4 SC oligonucleotides at 0.4

$\mu$ M dose in the presence or absence of doxorubicin (DOX), taxol, vinorelbine (VNB) or etoposide (Etop), as determined by MTT assay. The data represent the mean  $\pm$  SD of three independent experiments.

Fig. 14 is a graph showing relative H460 tumor growth in mice treated with XIAP AS 2x2 MBOs and vinorelbine. Intratumoral injection of oligonucleotides at 50  $\mu$ g/g tumor mass was performed in SCID-RAG2 mice carrying subcutaneous H460 cell xenografts. This treatment was combined with administration of vinorelbine.

Fig. 15 is a graph showing mean H460 cell tumor size in mice treated systemically with XIAP AS PS-oligonucleotides. Systemic delivery (i.p.) of XIAP AS PS-oligonucleotides into SCID-RAG2 mice implanted with subcutaneous H460 cell xenografts reduced the size of the tumors, relative to control.

Fig. 16 is a graph showing MDA-MB-435/LCC6 human breast carcinoma cell (LCC cell) tumor size in mice treated systemically with XIAP AS PS-oligonucleotides. Systemic delivery (i.p.) of XIAP AS PS-oligonucleotides into female SCID-RAG2 mice implanted with LCC6 cell xenografts in mammary fat pads reduced the size of the tumors, relative to control.

Fig. 17 is a schematic illustration showing *in vivo* effects of G4 oligonucleotides on tumor growth and tumor XIAP protein levels. Antitumor efficacy of systemically delivered, naked XIAP G4 AS oligonucleotides or G4 SC oligonucleotides on the growth of subcutaneous H460 cell xenografts in male SCID-RAG2 mice. All data are expressed as mean  $\pm$  SEM (n = 6 mice/group).

Figs. 18A and 18B are schematic illustrations depicting XIAP protein expression levels in H460 tumor xenografts implanted in SCID-RAG2 mice after 21 days treatment with G4 AS oligonucleotides, G4 SC oligonucleotides, or vehicle alone (control), analyzed by western blotting and quantified by densitometry. XIAP levels were normalized to cellular actin levels. All data are expressed as mean  $\pm$  SD (n = 3).

Figs. 19A and 19B are photomicrographs showing *in vivo* effects of G4 oligonucleotides on histopathology of H460 tumors implanted in SCID-RAG2 mice after 15 mg/kg systemic dosing of XIAP G4 AS oligonucleotides or G4 SC oligonucleotides over 21 days. Fig. 19A depicts tumor sections stained with hematoxylin and eosin. Fig. 19B shows immunohistochemistry of ubiquitin expression in tumor sections. Representative tumor photomicrographs are shown. Internal scale markers equal 100  $\mu\text{m}$ .

Figs. 20A and 20B are graphs showing increased *in vivo* efficacy of vinorelbine (VNB) in combination with XIAP oligonucleotides. Antitumor efficacy of VNB with or without XIAP G4 AS oligonucleotides or G4 SC oligonucleotides against H460 tumors xenografts was determined in SCID-RAG2 mice. Fig. 20A depicts antitumor activity of single agents, while Fig. 20B depicts antitumor activity of VNB and G4 oligonucleotides in combination. All data are expressed as means  $\pm$  SEM (n = 6 mice/group).

Fig. 21 is a graph showing the effects of HIAP1 oligonucleotides on HIAP1 RNA levels.

Figs. 22A and 22B are schematic illustrations showing densitometric scans of western blots showing the effects of HIAP1 oligonucleotides on a cell's ability to block cycloheximide-induced upregulation of HIAP1 protein.

Fig. 23 is a graph showing the effects of HIAP1 oligonucleotides on cytotoxicity, as measured by total protein.

Fig. 24 is a graph showing the validation of the sequence specificity for HIAP1 oligonucleotide APO 2.

Fig. 25 is a graph showing the effect of HIAP1 oligonucleotides on the chemosensitization of drug-resistant SF295 glioblastomas.

### Detailed Description of the Invention

The present invention provides nucleobase oligomers that inhibit expression of an IAP, and methods for using them to induce apoptosis in a cell. The nucleobase oligomers of the present invention may also be used to form pharmaceutical compositions. The invention also features methods for enhancing apoptosis in a cell by administering an oligonucleotide of the invention in combination with a chemotherapeutic agent such as a cytotoxic agent, cytostatic agent, or biological response modifying agent (e.g., adriamycin, vinorelbine, etoposide, taxol, cisplatin, interferon, interleukin-2, monoclonal antibodies). The chemotherapeutic agent may be, for example, a chemosensitizer (i.e., an agent that makes the proliferating cells more sensitive to the chemotherapy) may also be administered. Any combination of the foregoing agents may also be used to form a pharmaceutical composition. These pharmaceutical compositions may be used to treat a proliferative disease, for example, cancer or a lymphoproliferative disorder, or a symptom of a proliferative disease. The nucleobase oligomer of the invention may also be used in combination with radiotherapy for the treatment of cancer or other proliferative disease.

Activation of apoptosis in cancer cells offers novel, and potentially useful approaches to improve patient responses to conventional chemotherapy or radiotherapy. XIAP is the most potent member of the IAP gene family in terms of its ability to directly inhibit caspases and to suppress apoptosis. We investigated the effect of XIAP down-regulation by antisense (AS) oligonucleotides on human non-small cell lung cancer (NIH-H460) growth *in vitro* and *in vivo*. In cultured H460 human lung cancer cells, oligonucleotide G4 AS was identified as the most potent compound, effectively down-regulated XIAP mRNA by 55% and protein levels up to 60%, as determined by real-time RT-PCR and western blotting, respectively, and induced 60% cell death at 1.2  $\mu$ M concentrations. In contrast, the scrambled control G4 oligonucleotide caused little XIAP

loss and less than 10% cell death. Treatment with G4 AS induced apoptosis, as revealed by degradation of pro-caspase-3 and PARP proteins, with significant nuclear DNA condensation and fragmentation at 1.2  $\mu$ M concentrations. Moreover, XIAP AS oligonucleotides sensitized H460 cells to the cytotoxic effects of doxorubicin, taxol, vinorelbine, and etoposide. In animal models, we demonstrate that G4 AS at 15 mg/kg had significant sequence-specific growth inhibitory effects on human H460 tumors in xenograft models of SCID/RAG2-immunodeficient mice by systemic intraperitoneal administration. Systemic AS ODN administration was associated with an 85% down-regulation of XIAP protein in tumor xenografts. The combination of 15 mg/kg G4 AS with 5 mg/kg vinorelbine significantly inhibited tumor growth, more than either agent alone. These studies indicate that down-regulation of XIAP is a potent death signal in lung carcinoma cells and is able to induce apoptosis *in vitro* as well as inhibit tumor growth *in vivo*. These studies support the contention that IAPs are suitable targets for cancer therapy in human non-small cell lung cancer, as well as other solid tumors.

## Therapy

Therapy may be provided wherever cancer therapy is performed: at home, the doctor's office, a clinic, a hospital's outpatient department, or a hospital. Treatment generally begins at a hospital so that the doctor can observe the therapy's effects closely and make any adjustments that are needed. The duration of the therapy depends on the kind of cancer being treated, the age and condition of the patient, the stage and type of the patient's disease, and how the patient's body responds to the treatment. Drug administration may be performed at different intervals (e.g., daily, weekly, or monthly). Therapy may be given in on-and-off cycles that include rest periods so that the patient's body has a chance to build healthy new cells and regain its strength.



Depending on the type of cancer and its stage of development, the therapy can be used to slow the spreading of the cancer, to slow the cancer's growth, to kill or arrest cancer cells that may have spread to other parts of the body from the original tumor, to relieve symptoms caused by the cancer, or to prevent cancer in the first place.

As used herein, the terms "cancer" or "neoplasm" or "neoplastic cells" is meant a collection of cells multiplying in an abnormal manner. Cancer growth is uncontrolled and progressive, and occurs under conditions that would not elicit, or would cause cessation of, multiplication of normal cells.

A nucleobase oligomer of the invention, or other negative regulator of the IAP anti-apoptotic pathway, may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the compounds to patients suffering from a disease that is caused by excessive cell proliferation. Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intratumoral, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intrahepatic, intracapsular, intrathecal, intracisternal, intraperitoneal, intranasal, aerosol, suppository, or oral administration. For example, therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington: The Science and Practice of Pharmacy" Ed. A.R. Gennaro, Lippincourt Williams & Wilkins, Philadelphia, PA, 2000. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such

as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for IAP modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

The formulations can be administered to human patients in therapeutically effective amounts (e.g., amounts which prevent, eliminate, or reduce a pathological condition) to provide therapy for a disease or condition. The preferred dosage of a nucleobase oligomer of the invention is likely to depend on such variables as the type and extent of the disorder, the overall health status of the particular patient, the formulation of the compound excipients, and its route of administration.

As described above, if desired, treatment with a nucleobase oligomer of the invention may be combined with therapies for the treatment of proliferative disease (e.g., radiotherapy, surgery, or chemotherapy).

For any of the methods of application described above, a nucleobase oligomer of the invention is desirably administered intravenously or is applied to the site of the needed apoptosis event (e.g., by injection).

### **Oligonucleotides and other nucleobase oligomers**

At least two types of oligonucleotides induce the cleavage of RNA by RNase H: polydeoxynucleotides with phosphodiester (PO) or phosphorothioate (PS) linkages. Although 2'-OMe-RNA sequences exhibit a high affinity for RNA targets, these

sequences are not substrates for RNase H. A desirable oligonucleotide is one based on 2'-modified oligonucleotides containing oligodeoxynucleotide gaps with some or all internucleotide linkages modified to phosphorothioates for nuclease resistance. The presence of methylphosphonate modifications increases the affinity of the oligonucleotide for its target RNA and thus reduces the  $IC_{50}$ . This modification also increases the nuclease resistance of the modified oligonucleotide. It is understood that the methods and reagents of the present invention may be used in conjunction with any technologies that may be developed, including covalently-closed multiple antisense (CMAS) oligonucleotides (Moon et al., Biochem J. 346:295-303, 2000; PCT Publication No. WO 00/61595), ribbon-type antisense (RiAS) oligonucleotides (Moon et al., J. Biol. Chem. 275:4647-4653, 2000; PCT Publication No. WO 00/61595), and large circular antisense oligonucleotides (U.S. Patent Application Publication No. US 2002/0168631 A1).

As is known in the art, a nucleoside is a nucleobase-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric structure can be further joined to form a circular structure; open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred nucleobase oligomers useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, nucleobase oligomers having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone are also considered to be nucleobase oligomers.

Nucleobase oligomers that have modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity, wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

Nucleobase oligomers having modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic

internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts. Representative United States patents that teach the preparation of the above oligonucleotides include, but are not limited to, U.S. Patent Nos.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

In other nucleobase oligomers, both the sugar and the internucleoside linkage, i.e., the backbone, are replaced with novel groups. The nucleobase units are maintained for hybridization with an IAP. One such nucleobase oligomer, is referred to as a Peptide Nucleic Acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Methods for making and using these nucleobase oligomers are described, for example, in "Peptide Nucleic Acids: Protocols and Applications" Ed. P.E. Nielsen, Horizon Press, Norfolk, United Kingdom, 1999. Representative United States patents that teach the preparation of PNAs include, but are not limited to, U.S. Patent Nos.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

In particular embodiments of the invention, the nucleobase oligomers have phosphorothioate backbones and nucleosides with heteroatom backbones, and in particular  $-\text{CH}_2\text{-NH-O-CH}_2-$ ,  $-\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2-$  (known as a methylene (methylimino) or MMI backbone),  $-\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2-$ ,  $-\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2-$ , and  $-\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2-$ . In other embodiments, the oligonucleotides have morpholino backbone structures described in U.S. Patent No. 5,034,506.

Nucleobase oligomers may also contain one or more substituted sugar moieties. Nucleobase oligomers comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl, and alkynyl may be substituted or unsubstituted  $\text{C}_1$  to  $\text{C}_{10}$  alkyl or  $\text{C}_2$  to  $\text{C}_{10}$  alkenyl and alkynyl. Particularly preferred are  $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{OCH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{NH}_2$ ,  $\text{O}(\text{CH}_2)_n\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{ONH}_2$ , and  $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$ , where n and m are from 1 to about 10. Other preferred nucleobase oligomers include one of the following at the 2' position:  $\text{C}_1$  to  $\text{C}_{10}$  lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl, or O-aralkyl, SH,  $\text{SCH}_3$ , OCN, Cl, Br, CN,  $\text{CF}_3$ ,  $\text{OCF}_3$ ,  $\text{SOCH}_3$ ,  $\text{SO}_2\text{CH}_3$ ,  $\text{ONO}_2$ ,  $\text{NO}_2$ ,  $\text{NH}_2$ , heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of a nucleobase oligomer, or a group for improving the pharmacodynamic properties of an nucleobase oligomer, and other substituents having similar properties. Preferred modifications are 2'-O-methyl and 2'-methoxyethoxy ( $2'\text{-O-CH}_2\text{CH}_2\text{OCH}_3$ , also known as 2'-O-(2-methoxyethyl) or 2'-MOE). Another desirable modification is 2'-dimethylaminooxyethoxy (i.e.,  $\text{O}(\text{CH}_2)_2\text{ON}(\text{CH}_3)_2$ ), also known as 2'-DMAOE. Other modifications include, 2'-aminopropoxy ( $2'\text{-OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ ) and 2'-fluoro ( $2'\text{-F}$ ). Similar modifications may also be made at other positions on an oligonucleotide or other nucleobase oligomer, particularly the 3' position of the sugar on the 3' terminal

nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Nucleobase oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patent Nos.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference in its entirety.

Nucleobase oligomers may also include nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases, such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine; 2-propyl and other alkyl derivatives of adenine and guanine; 2-thiouracil, 2-thiothymine and 2-thiocytosine; 5-halouracil and cytosine; 5-propynyl uracil and cytosine; 6-azo uracil, cytosine and thymine; 5-uracil (pseudouracil); 4-thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines; 5-halo (e.g., 5-bromo), 5-trifluoromethyl and other 5-substituted uracils and cytosines; 7-methylguanine and 7-methyladenine; 8-azaguanine and 8-azaadenine; 7-deazaguanine and 7-deazaadenine; and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press,

1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of an antisense oligonucleotide of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines, and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2.degree. C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are desirable base substitutions, even more particularly when combined with 2'-O-methoxyethyl or 2'-O-methyl sugar modifications. Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include U.S. Patent Nos.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,681,941; and 5,750,692, each of which is herein incorporated by reference.

Another modification of a nucleobase oligomer of the invention involves chemically linking to the nucleobase oligomer one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 86:6553-6556, 1989), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 4:1053-1060, 1994), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 660:306-309, 1992; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 3:2765-2770, 1993), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 20:533-538: 1992), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 10:1111-1118, 1991; Kabanov et al., *FEBS Lett.*, 259:327-330, 1990; Svinarchuk et al., *Biochimie*, 75:49-54, 1993), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-



H-phosphonate (Manoharan et al., Tetrahedron Lett., 36:3651-3654, 1995; Shea et al., Nucl. Acids Res., 18:3777-3783, 1990), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 14:969-973, 1995), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 36:3651-3654, 1995), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1264:229-237, 1995), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 277:923-937, 1996). Representative United States patents that teach the preparation of such nucleobase oligomer conjugates include U.S. Patent Nos.: 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,828,979; 4,835,263; 4,876,335; 4,904,582; 4,948,882; 4,958,013; 5,082,830; 5,109,124; 5,112,963; 5,118,802; 5,138,045; 5,214,136; 5,218,105; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,414,077; 5,416,203; 5,451,463; 5,486,603; 5,510,475; 5,512,439; 5,512,667; 5,514,785; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,565,552; 5,567,810; 5,574,142; 5,578,717; 5,578,718; 5,580,731; 5,585,481; 5,587,371; 5,591,584; 5,595,726; 5,597,696; 5,599,923; 5,599,928; 5,608,046; and 5,688,941, each of which is herein incorporated by reference.

The present invention also includes nucleobase oligomers that are chimeric compounds. "Chimeric" nucleobase oligomers are nucleobase oligomers, particularly oligonucleotides, that contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide. These nucleobase oligomers typically contain at least one region where the nucleobase oligomer is modified to confer, upon the nucleobase oligomer, increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the nucleobase oligomer may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA

duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of nucleobase oligomer inhibition of gene expression. Consequently, comparable results can often be obtained with shorter nucleobase oligomers when chimeric nucleobase oligomers are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region.

Chimeric nucleobase oligomers of the invention may be formed as composite structures of two or more nucleobase oligomers as described above. Such nucleobase oligomers, when oligonucleotides, have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include U.S. Patent Nos.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is herein incorporated by reference in its entirety.

The nucleobase oligomers used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The nucleobase oligomers of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include U.S. Patent Nos.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721;

4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The nucleobase oligomers of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound that, upon administration to an animal, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention can be prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in PCT publication Nos. WO 93/24510 or WO 94/26764.

The term "pharmaceutically acceptable salts" refers to salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., J. Pharma Sci., 66:1-19, 1977). The base addition salts of acidic compounds are prepared by contacting the free acid form with a sufficient amount

of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable

pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides and other nucleobase oligomers, suitable pharmaceutically acceptable salts include (i) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (ii) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (iii) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (iv) salts formed from elemental anions such as chlorine, bromine, and iodine.

The present invention also includes pharmaceutical compositions and formulations that include the nucleobase oligomers of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral, or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal, or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration.

**Locked nucleic acids**

Locked nucleic acids (LNAs) are nucleobase oligomers that can be employed in the present invention. LNAs contain a 2'O, 4'-C methylene bridge that restrict the flexibility of the ribofuranose ring of the nucleotide analog and locks it into the rigid bicyclic N-type conformation. LNAs show improved resistance to certain exo- and endonucleases and activate RNase H, and can be incorporated into almost any nucleobase oligomer. Moreover, LNA-containing nucleobase oligomers can be prepared using standard phosphoramidite synthesis protocols. Additional details regarding LNAs can be found in PCT publication No. WO 99/14226 and U.S. Patent Application Publication No. US 2002/0094555 A1, each of which is hereby incorporated by reference.

**Arabinonucleic acids**

Arabinonucleic acids (ANAs) can also be employed in methods and reagents of the present invention. ANAs are nucleobase oligomers based on D-arabinose sugars instead of the natural D-2'-deoxyribose sugars. Underivatized ANA analogs have similar binding affinity for RNA as do phosphorothioates. When the arabinose sugar is derivatized with fluorine (2' F-ANA), an enhancement in binding affinity results, and selective hydrolysis of bound RNA occurs efficiently in the resulting ANA/RNA and F-ANA/RNA duplexes. These analogs can be made stable in cellular media by a derivatization at their termini with simple L sugars. The use of ANAs in therapy is discussed, for example, in Damha et al., *Nucleosides Nucleotides & Nucleic Acids* 20: 429-440, 2001.

### **Delivery of nucleobase oligomers**

We demonstrate herein that naked oligonucleotides are capable on entering tumor cells and inhibiting IAP expression. Nonetheless, it may be desirable to utilize a formulation that aids in the delivery of oligonucleotides or other nucleobase oligomers to cells (see, e.g., U.S. Patents 5,656,611, 5,753,613, 5,785,992, 6,120,798, 6,221,959, 6,346,613, and 6,353,055, each of which is hereby incorporated by reference).

### **Ribozymes**

Catalytic RNA molecules or ribozymes that include an antisense IAP sequence of the present invention can be used to inhibit expression of an IAP polynucleotide *in vivo*. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff et al., Nature 334:585-591, 1988, and U.S. Patent Application Publication No. 2003/0003469 A1, each of which is incorporated by reference.

Accordingly, the invention also features a catalytic RNA molecule that includes, in the binding arm, an antisense RNA having between eight and nineteen consecutive nucleobases corresponding to a sequence of any one of Tables 1, 2, 6, and 7. In preferred embodiments of this invention, the catalytic nucleic acid molecule is formed in a hammerhead or hairpin motif, but may also be formed in the motif of a hepatitis delta virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA. Examples of such hammerhead motifs are described by Rossi et al., Aids Research and Human Retroviruses, 8:183, 1992. Example of hairpin motifs are described by Hampel et al., "RNA Catalyst for Cleaving Specific RNA Sequences," filed Sep. 20, 1989, which is a continuation-in-part of U.S. Ser. No. 07/247,100 filed Sept. 20, 1988, Hampel and Tritz, Biochemistry, 28:4929, 1989, and Hampel et al., Nucleic Acids

Research, 18: 299, 1990. An example of the hepatitis delta virus motif is described by Perrotta and Been, *Biochemistry*, 31:16, 1992. The RNaseP motif is described by Guerrier-Takada et al., *Cell*, 35:849, 1983. The Neurospora VS RNA ribozyme motif is described by Collins et al. (Saville and Collins, *Cell* 61:685-696, 1990; Saville and Collins, *Proc. Natl. Acad. Sci. USA* 88:8826-8830, 1991; Collins and Olive, *Biochemistry* 32:2795-2799, 1993). These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

### **RNA interference**

The nucleobase oligomers of the present invention may be employed in double-stranded RNAs for RNA interference (RNAi)-mediated knock-down of IAP expression. RNAi is a method for decreasing the cellular expression of specific proteins of interest (reviewed in Tuschl, *ChemBiochem* 2:239-245, 2001; Sharp, *Genes & Devel.* 15:485-490, 2000; Hutvagner and Zamore, *Curr. Opin. Genet. Devel.* 12:225-232, 2002; and Hannon, *Nature* 418:244-251, 2002). In RNAi, gene silencing is typically triggered post-transcriptionally by the presence of double-stranded RNA (dsRNA) in a cell. This dsRNA is processed intracellularly into shorter pieces called small interfering RNAs (siRNAs). The introduction of siRNAs into cells either by transfection of dsRNAs or through expression of siRNAs using a plasmid-based expression system is increasingly being used to create loss-of-function phenotypes in mammalian cells.

In one embodiment of the invention, double-stranded RNA (dsRNA) molecule is made that includes between eight and nineteen consecutive nucleobases of a nucleobase



oligomer of the invention. The dsRNA can be two distinct strands of RNA that have duplexed, or a single RNA strand that has self-duplexed (small hairpin (sh)RNA). Typically, dsRNAs are about 21 or 22 base pairs, but may be shorter or longer (up to about 29 nucleobases) if desired. dsRNA can be made using standard techniques (e.g., chemical synthesis or *in vitro* transcription). Kits are available, for example, from Ambion (Austin, TX) and Epicentre (Madison, WI). Methods for expressing dsRNA in mammalian cells are described in Brummelkamp et al. *Science* 296:550-553, 2002; Paddison et al. *Genes & Devel.* 16:948-958, 2002. Paul et al. *Nature Biotechnol.* 20:505-508, 2002; Sui et al. *Proc. Natl. Acad. Sci. USA* 99:5515-5520, 2002; Yu et al. *Proc. Natl. Acad. Sci. USA* 99:6047-6052, 2002; Miyagishi et al. *Nature Biotechnol.* 20:497-500, 2002; and Lee et al. *Nature Biotechnol.* 20:500-505 2002,, each of which is hereby incorporated by reference.

Small hairpin RNAs consist of a stem-loop structure with optional 3' UU-overhangs. While there may be variation, stems can range from 21 to 31 bp (desirably 25 to 29 bp), and the loops can range from 4 to 30 bp (desirably 4 to 23 bp). For expression of shRNAs within cells, plasmid vectors containing either the polymerase III H1-RNA or U6 promoter, a cloning site for the stem-looped RNA insert, and a 4-5-thymidine transcription termination signal can be employed. The Polymerase III promoters generally have well-defined initiation and stop sites and their transcripts lack poly(A) tails. The termination signal for these promoters is defined by the polythymidine tract, and the transcript is typically cleaved after the second uridine. Cleavage at this position generates a 3' UU overhang in the expressed shRNA, which is similar to the 3' overhangs of synthetic siRNAs. Additional methods for expressing the shRNA in mammalian cells are described in the references cited above.

The following examples are to illustrate the invention. They are not meant to limit the invention in any way.

**Example 1: Nucleobase oligomer selection**

We selected 96 and 98, mostly non-overlapping, 19-mer nucleobase sequences for human XIAP and HIAP1, respectively, based on the selection criteria listed below. In the case of XIAP, we selected 96 sequences (each being 19 nucleobases in length) (SEQ ID NOs: 1 through 96; Table 1), from a region approximately 1 kb upstream of the start codon to approximately 1 kb downstream of the stop codon of the cDNA sequence. This blanketed approximately 50% of the coding region, and immediate 5' and 3' UTR sequences (i.e., 96 19-mers span 1.8 kb of sequence, while the targeted region is approximately 3.5 kb in length, comprised of a coding region of 1.5 kb plus 1 kb at either side of UTR sequences).

Table 1

SEQ ID NO:	Code	Nucleobase Sequence	XIAP down-regulation T24 RNA	XIAP down-regulation T24 protein	XIAP down-regulation H460 RNA
1	A1	AAAATTCTAAGTACCTGCA	-	-	48%
2	B1	TCTAGAGGGTGGCTCAGGA	-	-	66%
3	C1	CAGATATATATGTAACACT	-	-	66%
4	D1	TGAGAGCCCTTTTTTTGTT	-	-	75%
5	E1	AGTATGAAATAATTTCTGAT	-	-	69%
6	F1	ATTGGTTCCAATGTGTCT	-	-	81%
7	G1	TTAGCAAAATATGTTTTAA	-	-	33%
8	H1	TGAATTAAATTTTAATATC	-	-	13%
9	A2	ATTCAAAGGCATCAAAGTTG	-	-	58%
10	B2	GTCAAATCATTAATTAGGA	-	-	55%
11	C2	AATATGTAAACTGTGATGC	36%	45%	70%
12	D2	GCAGAAATAAACTAATAAT	-	-	39%
13	E2	GAAAGTAATATTTAAGCAG	54%	51%	60%
14	F2	TTACCACATCATTCGAAGTC	-	-	34%
15	G2	CTAAATACTAGAGTTCGAC	-	-	55%
16	H2	ACACGACCGCTAAGAAACA	-	-	46%
17	A3	TATCCACTTATGACATAAA	-	-	27%
18	B3	GTTATAGGAGCTAACAAAT	-	-	34%

SEQ ID NO:	Code	Nucleobase Sequence	XIAP down-regulation T24 RNA	XIAP down-regulation T24 protein	XIAP down-regulation H460 RNA
19	C3	AATGTGAAACACAAAGCAAC	-	-	43%
20	D3	ACATTATATTAGGAAATCC	-	-	30%
21	E3	CTTGTCACCTTTTCTAAA	53%	64%	55%
22	F3	ATCTTCTCTTGAAAATAGG	44%	53%	-
23	G3	CCTTCAAAACTGTAAAAAG	-	-	-
24	H3	ATGCTGCGAGGTACACAAAG	-	-	-
25	A4	ATCTATTAAACTCTTCTAC	-	-	-
26	B4	ACAGGACTACCACCTTGGAA	-	-	76%
27	C4	TGCCAGTGTGATGCTGAA	28%	56%	77%
28	D4	GTATAAAGAAACCCCTGCTC	12%	43%	51%
29	E4	CGCACGGTATCTCCTTCAC	47%	34%	51%
30	F4	CTACAGCTGCATGACAACT	33%	43%	-
31	G4	GCTGAGTCTCCATATTGCC	34%	48%	51%
32	H4	ATACTTTCCTGTGCTTCC	-	-	-
33	A5	GATAAATCTGCAATTGCG	-	-	-
34	B5	TTGTAGACTGCGTGGCACT	-	-	61%
35	C5	ACCATTCTGGATACCAGAA	71%	54%	-
36	D5	AGTTTCAACTTTGTACTG	39%	33%	-
37	E5	ATGATCTCTGCTTCCCAGA	-	-	46%
38	F5	AGATGGCCTGTCTAAGGCA	-	-	-

SEQ ID NO:	Code	Nucleobase Sequence	XIAP down-regulation T24 RNA	XIAP down-regulation T24 protein	XIAP down-regulation H460 RNA
39	G5	AGTTCTCAAAAGATAGTCT	-	-	30%
40	H5	GTGTCTGATATATCTACAA	-	-	39%
41	A6	TCGGGTATATGGTGTCTGA	-	-	72%
42	B6	CAGGGTTCCTCGGGTATAT	51%	47%	-
43	C6	GCTTCTTCACAATAACATGG	-	-	-
44	D6	GGCCAGTTCTGAAAGGACT	-	-	60%
45	E6	GCTAACTCTCTTGGGGTTA	-	-	-
46	F6	GTGTAGTAGAGTCCAGCAC	34%	39%	-
47	G6	AAGCACTGCACCTTGGTCAC	-	-	69%
48	H6	TTCAGTTTCCACCACAAC	-	-	68%
49	A7	ACGATCACAAGGTTCCCAA	-	-	-
50	B7	TCGCCCTGTGTTCTGACCAG	-	-	-
51	C7	CCGGCCCCAAAACAAGAAG	-	-	72%
52	D7	GATTCACCTTCGAATATTAA	56%	88%	46%
53	E7	TATCAGAACTCACAGCATC	-	-	-
54	F7	GGAAGATTGTGTAATTG	-	-	69%
55	G7	TCTGCCATGGATGGATTTC	-	-	41%
56	H7	AAGTAAAGATCCGTGCTTC	-	-	63%
57	A8	CTGAGTATATCCATGTCCC	-	-	-
58	B8	GCAAGCTGCTCCTTGTTAA	-	-	-

SEQ ID NO:	Code	Nucleobase Sequence	XIAP down-regulation T24 RNA	XIAP down-regulation T24 protein	XIAP down-regulation H460 RNA
59	C8	AAAGCATAAATCCAGCTC	-	-	16%
60	D8	GAAAGCACCTTACTTTATC	38%	26%	49%
61	H8	ACTGGGCTTCCAATCAGTT	-	-	-
62	E8	GTTGTTCCCAAGGGTCTTC	72%	56%	44%
63	F8	ACCCTGGATACCATTTAGC	-	-	47%
64	G8	TGTTCTAACAGATATTGTC	-	-	49%
65	A9	TATATAATCTTGTCCTTC	-	-	62%
66	B9	AGTTAAATGAATATTGTT	-	-	38%
67	C9	GACACTCCCTCAAGTGAATG	-	-	-
68	D9	TTTCTCAGTAGTTCTTACC	-	-	39%
69	E9	GTTAGTGATGGTGTTCCT	-	-	43%
70	F9	AGATGGTATCATCAATTCT	-	-	19%
71	G9	TGTACCATAGGATTTTGGGA	-	-	-
72	H9	CCCCATTGCTATAGCTTCT	-	-	-
73	A10	ATTATTTCTTAATGTCT	-	-	29%
74	B10	CAAGTGATTATAGTTGCT	-	-	-
75	C10	TAGATCTGCAACCAGAAACC	-	-	53%
76	D10	CATCTTGCACTACTGTCTTT	-	-	55%
77	E10	CCTTAGCTGCTCTTCAGTA	-	-	-
78	F10	AAGCTTCTCCTCTTGCAGG	-	-	51%

SEQ ID NO:	Code	Nucleobase Sequence	XIAP down-regulation T24 RNA	XIAP down-regulation T24 protein	XIAP down-regulation H460 RNA
79	G10	ATATTCTATCCATACAGA	-	-	56%
80	H10	CTAGATGTCCACAAGGAAC	-	-	-
81	A11	AGCACATTGTTTACAAGTG	-	-	68%
82	B11	AGCACATGGGACACTTGTC	-	-	63%
83	C11	CTTGAAAGTAATGACTGTG	-	-	52%
84	D11	CCTACTATAGAGTTAGATT	-	-	-
85	E11	ATTCAATCAGGGTAATAAG	-	-	48%
86	F11	AAGTCAGTTCACATCACAC	-	-	64%
87	G11	CAGTAAAAAAAATGGATAA	-	-	33%
88	H11	TTCAGTTATAGTATGATGC	-	-	-
89	A12	TACACTTAGAAAATTAAATC	-	-	46%
90	B12	TCCTCTATCTTCCACCAGC	-	-	-
91	C12	AGAA TCCTAAAAACACAACA	-	-	-
92	D12	ATTGCGACAAAGTACGTGTT	-	-	77%
93	E12	TGTCAGTACATGTTGGCTC	-	-	74%
94	F12	ACATAGTGTTTTGCCACTT	-	-	74%
95	G12	CTTTGATCTGGCTCAGACT	-	-	76%
96	H12	GAAACCACATTTAACAGTT	-	-	52%

Note that in any of the foregoing nucleobase oligomers, or any other nucleobase oligomers described herein, each nucleobase may independently be a DNA residue or RNA residue, such as a 2'-O-methyl or 2'-O-methoxyethyl RNA residue. For example, the nucleobase sequence of SEQ ID NO: 3 may be, for example, 5'-CAGATATATATGTAACACT-3', 5'-CAGATATATATGTAAACACU-3', or 5'-mCmAGATATATATGTAAcAmCmU-3' (wherein mX represents a 2'-O-methyl X residue). Additional modified nucleobases are known in the art. The linkages may be phosphodiester (PO), phosphorothioate (PS), or methylphosphonate (MP) linkages, or may have a mixed backbone (MB). The backbone may be any suitable backbone that allows hybridization of the nucleobase oligomer to the target IAP polynucleotide. Exemplary backbones are described herein. In other embodiments, the nucleobase oligomers include acridine-protected linkages, cholesteryl or psoralen components, C5-propynyl pyrimidines, or C5-methyl pyrimidines. Suitable modifications to the nucleobase oligomers of the invention include those described above, as well as those in U.S. Patent Application Publication No. US 2002/0128216 A1, hereby incorporated by reference.

Examples of nucleobase oligomers are provided in Table 2, below (wherein mX represents a 2'-O-methyl X RNA residue).

<b>Table 2</b>		
<b>2x2 MB PO</b>		<b>SEQ ID NO:</b>
DE4 as	mGmGTATCTCCTTCACCAGmUmA	97
DE4 rev	mAmUGACCACTTCCTCTATmGmG	98
δBC5 as	mGmATACCAGAATTmGmU	99
δBC5 rev	mUmGTTTAAGACCATmAmG	100
mG4 as	mGmCTGAGTCTCCATACTGmCmC	101
mG4 sm	mGmGCTCTCTGCCCACTGAmAmU	102
<b>3x3 MB PO</b>		
F3 as	mAmUmCTTCTCTTGAAAATmAmGmG	103
F3 scr	mCmAmGAGATTTTCATTTAAmCmGmU	104
F3 mm	mAmUmCTTGACTTGATTATmAmGmG	105



Table 2		
F3 rev	mGmGmATAAAAGTTCTCTTmCmUmA	106
E4 as	mCmGmCACGGTATCTCCTTmCmAmC	107
E4 scr	mCmUmACGCTCGCCATCGTmUmCmA	108
E4 rev	mCmAmCTTCCTCTATGGCmCmGmC	109
E4 mm	mCmGmCACCTATCTGGTmCmAmC	110
G4 as	mGmCmUGAGTCTCCATATTmGmCmC	111
G4 scr	mGmGmCTCTTTCGCCACTGmAmAmU	112
G4 rev	mCmCmGTTATACCTCTGAGmUmCmG	113
G4 mm	mGmCmUGACACTCCAATTTmGmCmC	114
C5 as	mAmCmCATTCTGGTAACCAmGmA	115
C5 scr	mUmGmCCCAAGAATACTAGmUmCmA	116
C5 mm	mAmCmCATAGTGGATTGCmGmA	117
C5 rev	mAmAmGACCATAGGTCTTAmCmCmA	118
D7 as	mGmAmUTCATTCTTCGAATATmUmAmA	119
D7 scr	mUmGmAAATGTAAATCATCmUmUmC	120
D7 mm	mGmAmUTCTGTTTCGATAATmUmAmA	121
D7 rev	mAmAmUTATAAGCTTCACTmUmAmG	122
<b>Phosphorothioate</b>		
PS-G4 as	GCTGAGTCTCCATATTGCC	123
PS-G4 sm	GGCTCTTTGCCCACTGAAT	124
PS-C5 as	ACCATTCTGGATACCAGAA	125
PS-C5 rev	AAGACCATAGGTCTTACCA	126
PS-F3 as	ATCTTCTCTTGAAAATAGG	127
PS-F3 rev	GGATAAAAGTTCTCTTCTA	128
PS-DE4 as	GGTATCTCCTTCACCAGTA	129
PS-DE4 rev	ATGACCACTTCCTCTATGG	130
PS-BC5 as	TCTGGATACCAGAATTTGT	131
PS-BC5 rev	TGTTTAAGACCATAGGTCT	132
PS-AB6 as	GGGTTCCCTCGGGTATATGG	133
PS-AB6 rs	GGTATATGGCGTCCTTGGG	134
PS-D7 as	GATTCACCTCGAATATTAA	135
PS-D7 rs	AATTATAACGTTCACCTAG	136
<b>Penetratin</b>		
F3 as	ATCTTCTCTTGAAAATAGG	137
G4 as	GCTGAGTCTCCATATTGCC	138
D7 as	GATTCACCTCGAATATTAA	139
C5 cs	TGCCCAAGAATACTAGTCA	140

<b>Table 2</b>		
<b>4X4 MBO PS (phosphorothioate linkages throughout)</b>		
G4 as	mGmCmUmGAGTCTCCATATmUmGmCmC	141
G4 sm	mGmGmCmUCTTTGCCCCACTmGmAmAmU	142
DE4 as	mGmGmUmATCTCCTTCACCMAmGmUmA	143
DE4 rev	mAmUmGmACCACTTCCTCTmAmUmGmG	144
E2 as	mGmAmAmAGTAATATTTAAmGmCmAmG	145
E2 rm	mGmAmGmCAATTTATAATGmAmAmAmG	146
H2G as	mAmCmCmGCTAAGAAACATmUmCmUmA	147
H2G rm	mAmUmCmUTACAAAGAATCmCmGmCmA	148
A3 as	mUmAmUmCCACTTATGACAmUmAmAmA	149
A3 rev	mAmAmAmUACAGTATTCACmCmUmAmU	150
FG8 as	mUmGmCmACCCTGGATACCMAmUmUmU	151
FG8 rm	mUmUmUmACCATAGGTCCCMAmGmCmU	152
mG4 as	mGmCmUmGAGTCTCCATACmUmGmCmC	153
mG4 sm	mGmGmCmUCTCTGCCCCACTmGmAmAmU	154
F1 as	mAmUmUmGGTTCCAATGTGmUmUmCmU	155
F1 rev	mUmCmUmUGTGTAACCTTGmGmUmUmA	156
B4 as	mAmCmAmGGACTACCACTTmGmGmAmA	157
B4 rev	mAmAmGmGTTCACCATCAGmGmAmCmA	158
G6 as	mAmAmGmCACTGCACTTGmUmCmAmC	159
G6 sm	mCmAmCmTGGTTGACCTCmCmAmAmG	160
E12 as	mUmGmUmCAGTACATGTTGmGmCmUmC	161
E12 sm	mCmUmAmGGTTGTCCATGAmCmUmGmU	162

Penetratin and its use in mediating entry of nucleobase oligomers into cells are described in PCT Patent Application No. FR 91/00444.

A similar identification approach was taken for designing nucleobase oligomers against HIAP1. Initially, 98 19-mer nucleobase oligomers were chosen (SEQ ID NOs: 163-260; Table 3). Of these 98 nucleobase oligomers targeted to the HIAP1 sequence, fifteen (SEQ ID NOs: 163-170, 173, 179, 202, 222, 223, 247, and 259) were selected for further evaluation. These fifteen candidate nucleobase oligomers included four nucleobase oligomers targeting the coding region (SEQ ID NOs: 202, 222, 223, and 247), one nucleobase oligomer targeting the 3' UTR (SEQ ID NO: 259), seven nucleobase oligomers targeting the 5' UTR (SEQ ID NOs: 166-170, 173, and 179; one of the seven

nucleobase oligomers overlapped the start codon), and three other oligonucleotides (SEQ ID NOs: 163-165) that were designed to target an intronic segment of the 5' UTR.

Table 3

SEQ ID NO:	Code	Nucleobase Oligomer Sequence
163	APO 1	TCATTGAGCCTGGGAGGT
164	APO 2	CGGAGGCTGAGGCAGGAGA
165	APO 3	GGTGTGGTGGTACGCGCCT
166	APO 4	ACCCATGCACAAAACCTACC
167	APO 5	AGAATGTGCCAGTAGGAGA
168	APO 6	TCTCACAGACGTTGGGCTT
169	APO 7	CCAGTGGTTTGCAAGCATG
170	APO 8	GAAATTTAGTGGCCAGGAA
171		AGAAATACACAATTGCACC
172		TACTGATACATTTTAAGGA
173	APO 9	TTCAACATGGAGATTCTAA
174		ATTTCTATGCATTTAGAGT
175		AATACTAGGCTGAAAAGCC
176		GGCTTTGCTTTTATCAGTT
177		TCTAGGGAGGTAGTTTTGT
178		GGGAAGAAAAGGGACTAGC
179	APO 10	G TTCATAATGAAATGAATG
180		ATAAGAATATGCTGTTTTC
181		TTCAAACGTGTTGGCGCTT
182		ATGACAAGTCGTATTTTCTAG
183		AAGTGGAATACGTAGACAT
184		AGACAGGAACCCAGCAGG
185		CGAGCAAGACTCCTTTCTG
186		AGTGTAATAGAAACCAGCA
187		TGACCTTGTCATTACACC
188		TTATCCAGCATCAGGCCAC
189		ACTGTCTCCTCTTTTCCAG
190		TTTTATGCTTTTTCAGTAGG
191		ACGAATCTGCAGCTAGGAT
192		CAAGTTGTAAACGGAATTT
193		TAGGCTGAGAGGTAGCTTC
194		GTTACTGAAGAAGGAAAAG
195		GAATGAGTGTGTGGAATGT

SEQ ID NO:	Code	Nucleobase Oligomer Sequence
196		TGTTTTCTGTACCCGGAAG
197		GAGCCACGGAAATATCCAC
198		TGATGGAGAGTTTGAATAA
199		GATTTGCTCTGGAGTTTAC
200		GGCAGAAAATTCTTGATTT
201		GGACAGGGGTAGGAACTTC
202	APO 11	GCATTTTCGTTATTCATTG
203		CTGAAAAGTAAGTAATCTG
204		GGCGACAGAAAAGTCAATG
205		CCACTCTGTCTCCAGGTCC
206		CCACCACAGGCAAAGCAAG
207		TTCGGTTCCCAATTGCTCA
208		TTCTGACATAGCATTATCC
209		TGGGAAAATGTCTCAGGTG
210		TATAAATGGGCATTTGGGA
211		TGTCTTGAAGCTGATTTTC
212		GAAACTGTGTATCTTGAAG
213		TGTCTGCATGCTCAGATTA
214		GAATGTTTTAAAGCGGGCT
215		CACTAGAGGGCCAGTTAAA
216		CCGCACTTGCAAGCTGCTC
217		CATCATCACTGTTACCCAC
218		CCACCATCACAGCAAAAGC
219		TCCAGATTCCCAACACCTG
220		CCCATGGATCATCTCCAGA
221		AACCACTTGGCATGTTGAA
222	APO 12	CAAGTACTCACACCTTGGA
223	APO 13	CCTGTCCTTTAATTCTTAT
224		TGAACTTGACGGATGAACT
225		TAGATGAGGGTAACTGGCT
226		TGGATAGCAGCTGTTCAAG
227		CATTTTCATCTCCTGGGCT
228		TGGATAATTGATGACTCTG
229		GTCTTCTCCAGGTTCAAAA
230		TATTCATCATGATTGCATC
231		CATTTCCACGGCAGCATT
232		CCAGGCTTCTACTAAAGCC
233		GCTAGGATTTTCTCTGAA
234		TCTATAATTCTCTCCAGTT

SEQ ID NO:	Code	Nucleobase Oligomer Sequence
235		ACACAAGATCATTGACTAG
236		TCTGCATTGAGTAAGTCTA
237		CTCTTCCCTTATTTTCATCT
238		TCCTCAGTTGCTCTTTCTC
239		GCCATTCTATTCTTCCGGA
240		AGTCAAATGTTGAAAAAGT
241		CCAGGATTGGAATTACACA
242		ATTCCGGCAGTTAGTAGAC
243		TAACATCATGTTCTTGTTTC
244		GTCTGTGTCTTCTGTTTAA
245		TTCTCTTGCTTGTAAGAC
246		CTAAAATCGTATCAATCAG
247	APO 14	GGCTGCAATATTTCTTTT
248		GAGAGTTTCTGAATACAGT
249		ACAGCTTCAGCTTCTTGCA
250		AAATAAATGCTCATATAAC
251		GAAACATCTTCTGTGGGAA
252		GTTCTTCCACTGGTAGATC
253		CTTCTTGTAAGTCTCCGCAA
254		TTGTCCATACACACTTTAC
255		AACCAAATTAGGATAAAAG
256		ATGTTTCATATGGTTTAGAT
257		TAAGTTTTACTTCACTTAC
258		ATGTTCCCGGTATTAGTAC
259	APO 15	GGGCTCAAGTAATTCTCTT
260		GCCCAGGATGGATTCAAAC

#### Nucleobase oligomer selection criteria

The computer program OLIGO (previously distributed by National Biosciences Inc.) was used to select candidate nucleobase oligomers based on the following criteria:

- 1) no more than 75% GC content, and no more than 75% AT content;
- 2) preferably no nucleobase oligomers with four or more consecutive G residues (due to reported toxic effects, although one was chosen as a toxicity control);

3) no nucleobase oligomers with the ability to form stable dimers or hairpin structures; and

4) sequences around the translation start site are a preferred region.

In addition, accessible regions of the target mRNA were predicted with the help of the RNA secondary structure folding program MFOLD (M. Zuker, D.H. Mathews & D.H. Turner, Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In: *RNA Biochemistry and Biotechnology*, J. Barciszewski & B.F.C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, (1999). Sub-optimal folds with a free energy value within 5% of the predicted most stable fold of the mRNA were predicted using a window of 200 bases within which a residue can find a complimentary base to form a base pair bond. Open regions that did not form a base pair were summed together with each suboptimal fold and areas that consistently were predicted as open were considered more accessible to the binding to nucleobase oligomers. Additional nucleobase oligomer that only partially fulfilled some of the above selection criteria were also chosen as possible candidates if they recognized a predicted open region of the target mRNA.

### **Example 2: Oligonucleotide synthesis**

The ability of nucleobase oligomers to inhibit IAP expression was tested using oligonucleotides as exemplary nucleobase oligomers. The oligonucleotides were synthesized by IDT (Integrated DNA Technologies, USA) as chimeric, second-generation oligonucleotides, consisting of a core of phosphodiester DNA residues flanked on either side by two 2'-O-methyl RNA residues with a phosphorothioate linkage between the flanking RNA residues. The oligonucleotides were provided in a 96-well plate, as well as matching tubes, with a minimum of 12 ODs of nucleobase oligomer, which provided ample material for transfections (greater than a hundred assays in the

96-well format) when the detection method is a sensitive method, such as TaqMan quantitative PCR, or an ELISA. Once the positive hits were identified (see below), oligonucleotides were re-synthesized with three, instead of two, flanking RNA residues to further increase stability/nuclease resistance. In addition, for validation purposes, appropriate controls (such as scrambled, 4-base mismatch, and reverse polarity oligonucleotides) were synthesized for some of the targets that yielded the highest activity.

### **Example 3: Screening assays and optimization of nucleobase oligomers**

Our approach to identifying nucleobase oligomers capable of inhibiting expression of an IAP was to screen the above-described oligonucleotide libraries for specific decreases (knock-down) of the RNA and/or protein for the specific IAP gene targeted. Any number of standard assays may be used to detect RNA and protein levels in cells. For example, RNA levels can be measured using standard northern blot analysis or RT-PCR techniques. Protein levels can be measured, for example, by standard western blot analyses or immunoprecipitation techniques. Alternatively, cells administered an antisense IAP nucleic acid may be examined for cell viability, according to methods described, for example, in U.S. Patent Nos. 5,919,912, 6,156,535, and 6,133,437, incorporated herein by reference.

We used TaqMan quantitative PCR (described below) to assay for changes in mRNA levels after oligonucleotide treatment. We employed ELISA for determining XIAP protein levels and western blotting for determining HIAP1 protein levels. Transfection conditions were optimized with Lipofectamine plus or Lipofectamine 2000 (Life Technologies, Canada) on T24 bladder carcinoma cells or H460 non-small cell lung carcinoma cells, or lipofectin on SF-295 glioblastoma cells, using a fluorescein-tagged sense oligonucleotide (5'-mGmAGAAGATGACTGGTAAmCmA-3'; SEQ ID NO: 261)

from XIAP spanning the start codon as a control. The results were visualized and gauged by epi-fluorescence microscopy. In the case of T24 cells, transfections were further optimized based on the ability of a published oligonucleotide to downregulate survivin expression (Li et al., Nat. Cell Biol. 1:461-466, 1999) (5'-U/TGTGCTATTCTGTGAAU/TU/T-3' SEQ ID NO: 262). We optimized the transfection conditions based on the TaqMan results of survivin RNA knock-down detected with PCR primers and fluorescent probe, described in detail below. Optimal conditions for oligonucleotide uptake by the cells were found to be 940 nM oligonucleotide and 4  $\mu$ L PLUS reagent and 0.8  $\mu$ L Lipofectamine in a total of 70  $\mu$ L for three hours. We then applied these conditions to screen for XIAP protein knock-down using the oligo library against T24 cells.

HIAP1 knock-down was studied in SF-295 cells because these cells had easily detectable and discernable 70 kDa HIAP1 protein, while many cell lines do not express high levels of the protein, or are not distinguishable from the large amounts of the similarly sized 68 kDa HIAP2 protein.

### **Real-time PCR**

RNA was extracted from cells lysed in RLT buffer (QIAGEN, Valencia, CA), and purified using QIAGEN RNeasy columns/kits. Real-time quantitative PCR was performed on a Perkin-Elmer ABI 7700 Prism PCR machine. RNA was reverse transcribed and amplified according to the TaqMan Universal PCR Master Mix protocol of PE Biosystems, using primers and probes designed to specifically recognize XIAP, HIAP1, survivin, or GAPDH. For human survivin, the forward primer was 5'-TCTGCTTCAAGGAGCTGGAA-3' (SEQ ID NO: 263), the reverse primer was 5'-GAAAGGAAAGCGCAACCG-3' (SEQ ID NO: 264), and the probe was 5'-(FAM)AGCCAGATGACGACCCCATAGAGGAACATA(TAMRA)-3' (SEQ ID NO: 265). For human HIAP1, the forward primer was 5'-TGGAGATGATCCATGGGTTCA-3' (SEQ ID NO: 266), the



reverse primer was 5'-GAACTCCTGTCCTTTAATTCTTATCAAGT-3' (SEQ ID NO: 267), and the probe was 5'-(FAM)CTCACACCTTGGAAACCACTTGGCATG (TAMRA)-3' (SEQ ID NO: 268). For human XIAP, the forward primer was 5'-GGTGA TAAAGTAAAGTGCTTTCACTGT-3' (SEQ ID NO: 269), the reverse primer was 5'-TCAGTAGTTCTTACCAGACACTCCTCAA-3' (SEQ ID NO: 270), and the probe was 5'-(FAM)CAACATGCTAAATGGTATCCAGGGTGCAAATATC(TAMRA)-3' (SEQ ID NO: 271). For human GAPDH, the forward primer was 5'-GAAGGTGAAGG TCGGAGTC-3' (SEQ ID NO: 272), the reverse primer was 5'-GAAGATGGTGTATGG GATTC-3' (SEQ ID NO: 273), and the probe was 5'-(JOE)CAAGCTTCCCGTTCTCA GCC(TAMRA)-3' (SEQ ID NO: 274). FAM is 6-carboxyfluorescein, JOE is 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein, and TAMRA is 6-carboxy-N,N,N',N'-tetramethylrhodamine. FAM and JOE are 5' reporter dyes, while TAMRA is a 3' quencher dye.

Relative quantification of gene expression was performed as described in the PE Biosystems manual using GAPDH as an internal standard. The comparative Ct (cycle threshold) method was used for relative quantitation of IAP mRNA levels compared to GAPDH mRNA levels. Briefly, real-time fluorescence measurements were taken at each PCR cycle and the threshold cycle (Ct) value for each sample was calculated by determining the point at which fluorescence exceeded a threshold limit of 30 times the baseline standard deviation. The average baseline value and the baseline SD are calculated starting from the third cycle baseline value and stopping at the baseline value three cycles before the signal starts to exponentially rise. The PCR primers and/or probes for the specific IAPs were designed to span at least one exon-intron boundary separated by 1 kb or more of genomic DNA, to reduce the possibility of amplifying and detecting genomic DNA contamination. The specificity of the signal, and possible contamination

from DNA, were verified by treating some RNA samples with either DNase or RNase, prior to performing the reverse transcription and PCR reaction steps.

#### **XIAP ELISA and HIAP1 western immunoblots**

A standard colorimetric XIAP ELISA assay was performed using an affinity-purified rabbit polyclonal antibody to XIAP as a capture antibody, and was detected with a XIAP monoclonal antibody (MBL, Japan) and a biotinylated anti-mouse Ig antibody and horseradish peroxidase-conjugated streptavidin and TMB substrate. Alternatively, a polyclonal XIAP or HIAP1 antibody may be used to measure XIAP or HIAP1 protein levels, respectively.

HIAP1 was detected on a western immunoblot using an affinity-purified anti-rat HIAP1 rabbit polyclonal antibody as a primary antibody and was detected by ECL (Amersham) on X-ray film with a secondary horseradish-peroxidase-conjugated anti-rabbit Ig antibody and a chemiluminescent substrate. The anti-HIAP1 polyclonal antibody is raised against a GST-fusion of the rat HIAP1. This antibody cross-reacts with both human and murine HIAP1 and HIAP2.

#### **Example 4: Antisense XIAP oligonucleotides decrease XIAP RNA and polypeptide expression**

The XIAP synthetic library of 96 antisense oligonucleotides was first screened for decreases in XIAP protein levels. Specifically, T24 cells ( $1.5 \times 10^4$  cells/well) were seeded in wells of a 96-well plate on day 1, and were cultured in antibiotic-free McCoy's medium for 24 hours. On day 2, the cells were transfected with XIAP antisense oligonucleotides as described above (oligonucleotides are labeled according to their plated position, i.e., A1 to H12, and include two repeats, A13 and B13 that contain lyophilized DNA pellets that stuck to the sealing membrane). Briefly, the nucleobase

oligomers were diluted in 10  $\mu$ l/well of serum-free, antibiotic-free McCoy's medium and then PLUS reagent was added. Lipofectamine was diluted in 10  $\mu$ l/well of serum-free, antibiotic-free McCoy's medium, and both mixes were incubated for 15 minutes at room temperature. The mixes were then combined and incubated for 15 minutes at room temperature.

In the meantime, the complete medium was removed from the cells and 50  $\mu$ l/well of serum-free, antibiotic-free medium was added to the cells. The transfection mixes were added to the well, and the cells were incubated for three hours. Then 30  $\mu$ l/well of serum-free, antibiotic-free medium and 100  $\mu$ l/well of antibiotic-free complete medium, containing 20% fetal bovine serum were added to each well.

At day 3, XIAP RNA levels were measured using quantitative real-time PCR techniques, as described above. At day 4, XIAP protein levels were measured by ELISA (Figs. 1A, 1C, 1E, 1G, 1I, and 1K), and total cellular protein was measured biochemically (Figs. 1B, 1D, 1F, 1H, 1J, and 1L; used to normalize the XIAP protein levels). The results were compared to a mock transfection sample (treated with the transfection agent but no oligonucleotide DNA was added, and then processed as for the other samples). Time course experiments determined that the optimal time for protein knock-down to be around 12 to 24 hours.

The oligonucleotide library was also screened for decreases in RNA levels, using TaqMan-specific PCR primers and fluorescent probes at the appropriate optimal time, using the primers and probes described above. Time course experiments determined mRNA to be optimally decreased at 6 to 9 hours. These results agree well with the protein results.

The first screen (although performed at a sub-optimal time point when XIAP levels are returning to normal, possibly due to an outgrowth of non-transfected cells) identified 16 antisense oligonucleotides (Table 1: C2, E2, E3, F3, C4, D4, E4, F4, G4,

C5, D5, B6, F6, D7, D8, F8) out of the 96 nucleobase oligomers tested that showed some decrease in XIAP protein levels relative to total protein, compared to mock (no nucleobase oligomer) transfection levels (Fig. 1A, 1C, 1E, 1G, 1I, and 1K). Total protein was decreased for each of these 16 nucleobase oligomers, which indicates a toxic or cytostatic effect of these nucleobase oligomers (Fig. 1B, 1D, 1F, 1H, 1J, 1L). Nucleobase oligomers B9 and C9 showed a clear drop in total protein but no relative drop in XIAP protein levels.

The 16 antisense nucleobase oligomers that showed some decrease in relative XIAP protein levels compared to mock transfection, were re-tested alone or in combination, with one control nucleobase oligomer (D2) included, for their ability to knock-down XIAP protein at a more optimal time point (12 hours) based on the above described time course studies (Fig. 2B). These nucleobase oligomers were also examined for their ability to decrease XIAP mRNA levels at 12 hours, normalized against GAPDH levels, and compared to mock transfection. Total protein concentrations at 12 hours were also determined (Fig. 2C).

There was a good correlation between the ability of a nucleobase oligomer to decrease XIAP protein levels (Fig. 2B) with its ability to decrease XIAP mRNA levels (Fig. 2A). In addition, there is no major loss of total protein at this early time point, and the decrease in XIAP mRNA and protein precede the decrease in total protein that is seen at later time points. The nucleobase oligomers that showed greater than 50% loss of XIAP protein or mRNA levels alone, or in a combination of two nucleobase oligomers added at a 1:1 ratio, were identified as the best nucleobase oligomers and validated further. Of these 16 oligonucleotides, ten (E2, E3, F3, E4, F4, G4, C5, B6, D7, F8) showed a consistent ability to decrease XIAP protein or RNA levels by more than 50%, depending on the transfection conditions used, or when used in combination (as for C5 and G4). Moreover, these 16 oligonucleotides that demonstrated antisense activity

clustered in four different target regions of the XIAP mRNA, with adjacent nucleobase oligomers showing some knock-down activity. Little or no antisense activity was observed with nucleobase oligomers that target sequences between these regions or islands of sensitivity. Presumably, these regions represent open areas on the mRNA that are accessible to nucleobase oligomers inside the cell. Two nucleobase oligomers, E3 and F3, target XIAP just upstream of the start codon in the intervening region between the IRES and the translation start site, and partially overlap the end of the IRES element. C2, D2, and E2 target a XIAP region upstream of the minimal IRES element, providing further evidence that the minimal IRES region is a highly structured region of RNA that is not readily accessible to nucleobase oligomers *in vivo*. All the other nucleobase oligomers are complimentary to a portion of the coding region, including a cluster of activity at positions 856-916 of the XIAP sequence (E4, F4, and G4) and smaller separate areas, as demonstrated by nucleobase oligomers C5 and D5, for example.

A portion of the 96 nucleobase oligomers depicted in Table 1 were rescreened for their ability to knock-down XIAP mRNA in NCI-H460 cells at 9 hours post-transfection. The data are summarized in Table 4, below.

Table 4

2x2 MBO	XIAP RNA	Std. Dev.	2x2 MBO	XIAP RNA	Std. Dev.
Untrf. Co.	1.04	0.055	A6 as	0.27	0
Mock Co.	1.01	0.006	D6 as	0.39	0.03
G4 sm	0.97	0.071	G6 as	0.3	0.01
DE4 rev	1.06	0.121	H6 as	0.31	0.01
A1 as	0.46	0.01	C7 as	0.27	0.02
B1 as	0.34	0.03	D7 as	0.52	0.04
C1 as	0.3	0.04	F7 as	0.3	0.04
D1 as	0.25	0.03	G7 as	0.66	0.04
E1 as	0.31	0.01	H7 as	0.49	0.01
F1 as	0.19	0.01	C8 as	1.01	0.08
G1 as	0.67	0.03	D8 as	0.55	0.04
H1 as	0.87	0.03	F8 as	0.62	0
A2 as	0.42	0.02	G8 as	0.64	0.06
B2 as	0.45	0.03	H8 as	0.61	0.06
C2 as	0.33	0.02	A9 as	0.46	0.02
D2 as	0.66	0.01	B9 as	0.74	0.07
E2 as	0.44	0.01	D9 as	0.73	0.04
F2 as	0.64	0.02	E9 as	0.69	0.06
G2 as	0.44	0.01	F9 as	0.97	0.15
H2 as	0.56	0.04	A10 as	0.85	0.04
A3 as	0.71	0.03	C10 as	0.56	0.01
B3 as	0.64	0.08	D10 as	0.54	0.01
C3 as	0.55	0.04	F10 as	0.64	0
D3 as	0.68	0.02	G10 as	0.49	0
E3 as	0.48	0.02	A11 as	0.36	0.03
B4 as	0.23	0.01	B11 as	0.39	0.02
C4 as	0.22	0.04	C11 as	0.44	0.03
D4 as	0.48	0.04	E11 as	0.52	0.04
E4 as	0.44	0.01	F11 as	0.36	0.05
G4 as	0.48	0.02	G11 as	0.67	0.02
B5 as	0.38	0.03	A12 as	0.54	0.03
E5 as	0.52	0.05	D12 as	0.23	0.05
G5 as	0.68	0.05	E12 as	0.26	0.01
H5 as	0.59	0.09	F12 as	0.26	0.03
			G12 as	0.24	0.05
			H12 as	0.48	0.06

We also determined whether 4X4 MBOs (all PS, DNA residues flanked on both sides by four 2'-O-methyl RNA residues) were capable of knocking-down XIAP protein in H460 cells. As shown in Figs. 3 and 4, 4X4 MBs of E12 and another oligonucleotide, FG8, were effective in amounts as low as 31 nM.

#### **Example 5: XIAP antisense nucleobase oligomers increase cytotoxicity and chemosensitization**

We investigated if XIAP antisense nucleobase oligomers could chemosensitize the highly drug resistant T24 cells to traditional chemotherapeutic agents, such as adriamycin or cisplatin. Antisense oligonucleotides were chosen to represent some of the different XIAP target regions and tested for their cytotoxic effects, alone or in combination with other oligonucleotides or drugs. Five XIAP antisense oligonucleotides were tested for their ability to kill or chemosensitize T24 bladder carcinoma cells, and were compared to the effects of three corresponding scrambled control oligonucleotides.

T24 cells were transfected with XIAP antisense oligonucleotides, scrambled oligonucleotides, no oligonucleotides (mock transfected), or were left untreated. The cells were tested for viability 20 hours after transfection (with the exception of the untreated control) using the WST-1 tetrazolium dye assay in which WST-1 tetrazolium dye is reduced to a colored formazan product in metabolically active cells (Fig. 5A).

The occurrence of cytotoxicity induced by oligonucleotide E4 was examined by visually inspecting T24 cells that were left untreated, mock transfected, or transfected with E4, E4 scrambled, E4 reverse polarity, or E4 mismatched oligonucleotides. Twenty hours after transfection, the cells were examined for morphology (Fig. 5D). Only the cell transfected with antisense E4 oligonucleotides showed signs of toxicity.

To examine the effects of the nucleobase oligomers on the chemosensitization of the T24 cells to cisplatin or adriamycin, oligonucleotides were tested for their ability to

further kill T24 cells in the presence of a fixed dose of adriamycin (0.5 µg/ml). Cells were first transfected with an oligonucleotide, then adriamycin was added for another 20 hours. Viability was measured by WST-1 at the end of the 20-hour drug treatment (Fig. 5B). Results are shown in Fig. 5C as percentage viability compared to nucleobase oligomer treatment alone.

All five nucleobase oligomers tested (F3, E4, G4, C5, D7) as well as combinations of E4+C5 and G4+C5, killed the T24 cells, leaving only 10-15% surviving cells after 24 hours, as compared to the mock (no oligonucleotide) transfected cells, or to cells transfected with three corresponding scrambled controls to F3 (5'-mCmAmGAGATTT CATTTAAmCmGmU-3'; SEQ ID NO: 275), E4 (5'-mCmUmACGCTCGCCATCGTmUmCmA-3'; SEQ ID NO: 276) and C5 (5'-mUmGmCCCAAGAATACTAGmUmCmA-3'; SEQ ID NO: 277)(Figs. 5A and 5C). Therefore, the toxicity is sequence-specific to those nucleobase oligomers that reduce XIAP levels, and not to a non-sequence specific toxicity due to nucleobase oligomers this chemistry in general. This cytotoxicity may result from the combined effect of XIAP protein knock-down (and the expected loss of anti-apoptotic protection afforded by XIAP) and the cytotoxicity of the transfection itself.

The addition of a fixed dose of adriamycin or cisplatin at the end of the three hour transfection period resulted in a further decrease in survival for some of the tested oligonucleotides, a further 40% drop in survival after 20 hours for nucleobase oligomers F3, D7 and G4+C5 combination (Fig. 5B), compared to their corresponding oligonucleotide-treated values (Fig. 5C). The values in Fig. 5B (oligonucleotide plus drug) are compared to the values of oligonucleotide alone in Fig. 5C, which is set a 100% for each ODN. Only the results for adriamycin chemosensitization are shown; similar results were obtained when the cells were chemosensitized with cisplatin. At the fixed doses used, the mock and scrambled control transfections did not show any increased loss



of survival when either treated with adriamycin (Fig. 5B). Chemosensitization is only seen when XIAP levels are decreased by a specific antisense oligonucleotide.

**Example 6: Down-regulating effects of antisense oligonucleotides on XIAP mRNA in H460 cells**

By using real-time PCR, antisense oligonucleotides (2x2 MBO, composed of two flanking 2'-O-methyl RNA residues at either end with phosphorothioate linkages, and a central core of 15 phosphodiester DNA residues) were examined for their effects on XIAP mRNA in H460 cells. In this configuration, nucleobase oligomers F3, G4, C5, AB6 and DE4 reduced the mRNA level by 50-70%, compared to untreated control, while D7 AS nucleobase oligomers reduced the mRNA level by 30% (Fig. 6). In contrast, control nucleobase oligomers and transfectant agent alone (LFA) each only reduced the mRNA level to less than 20% of untreated control (Fig. 6). Nucleobase oligomers F3, G4 and C5 were selected for further study *in vitro* and *in vivo*. Additional knockdown of XIAP mRNA observed by TaqMan analysis is depicted in Figs. 7 and 8.

**Example 7: Down-regulating effects of antisense oligonucleotides on XIAP protein**

We characterized the potency of nucleobase oligomers F3, G4 and C5 in oligonucleotide configuration on the XIAP protein expression by western blot analysis (Fig. 9, 10A, and 10B). G4 AS oligonucleotides exhibited the strongest down-regulating effect on XIAP protein, reducing XIAP protein levels by 62% at 24 h after the end of transfection at a concentration of 1.2  $\mu$ M (Figs. 10A and 10B). F3 AS oligonucleotides at 1.2  $\mu$ M reduced XIAP protein level by 50%, while C5 AS oligonucleotides did not show sequence specific effects compared to its control (Fig. 10B). In additional studies, E12 and FG8 AS oligonucleotides significantly reduced XIAP protein levels (Fig. 9).

**Example 8: Induction of apoptosis by XIAP AS oligonucleotides**

Having demonstrated that XIAP AS nucleobase oligomers were capable of reducing viability of H460 cells and T24 bladder carcinoma cells after, we determined whether the observed cell death was due to the induction of apoptosis. As shown in Fig. 11A, H460 cells treated with F3 or G4 AS oligonucleotides at 1.2  $\mu$ M activated and degraded pro-caspase-3 protein with a reduction of 40% or 60% of protein levels, respectively, compared to untreated control cells. PARP was also to its predicted caspase-3-generated fragment (Fig. 11A). In contrast, F3 and G4 SC oligonucleotide controls at 1.2  $\mu$ M did not have any effect on caspase-3 or PARP protein expression (Fig. 11A). The ratio of Bcl-2:Bax was unchanged in H460 cells treated with F3 and G4 AS oligonucleotides and their respective controls at 1.2  $\mu$ M. Flow cytometry was used to detect the hypo-diploid DNA content in H460 cells treated with G4 AS oligonucleotides and stained with PI (Fig. 12A). When H460 cells were treated with G4 AS oligonucleotides or scrambled control oligonucleotides at 1.2  $\mu$ M, the hypo-diploid DNA content of cells was 40.8 and 22.1%, respectively, compared to 16.6% for untreated control cells. DAPI staining was used to detect the nuclear morphological changes of the H460 cells treated with G4 AS oligonucleotides or scrambled control oligonucleotides at 1.2  $\mu$ M. As shown in Figure 12B, cells treated with G4 AS oligonucleotides underwent morphological changes characteristic of apoptosis, including chromatin condensation and nuclear DNA fragmentation. Few cells showed these morphological changes in G4 SC-treated control cells.

**Example 9: Inhibition of cell growth and sensitization of H460 cells to anticancer agents by AS oligonucleotides**

To analyze biological effects of nucleobase oligomers associated with down-regulation of XIAP expression and apoptosis, the growth of H460 cells treated with G4

AS oligonucleotides was investigated by MTT assay. Forty-eight hours after the transfection, G4 AS oligonucleotides had reduced H460 cell growth in a dose-dependent manner, exhibiting a 55% reduction relative to untreated control levels at 1.2  $\mu$ M (Fig. 13A). In contrast, the growth-inhibitory effect of G4 SC oligonucleotides, or transfectant agent alone, was comparatively low, only less than 10% of their untreated control.

To investigate whether down-regulation of XIAP expression has the potential to sensitize H460 cells to chemotherapy, combination treatments using G4 AS oligonucleotides and one of the following anticancer drugs: doxorubicin (DOX), taxol, vinorelbine (VNB) and etoposide (Etop) were performed. Fig. 13B demonstrates that each of the combinations resulted in at least an additive cytotoxic effect on the cell death, compared to treatment with either G4 AS oligonucleotides or the anticancer drugs alone.

#### **Example 10: Antitumor efficacy of G4 AS oligonucleotides on H460 and LCC6 tumor xenografts**

We first determined whether intra-tumoral injection of XIAP antisense 2x2-MBOs into SCID-RAG2 mice carrying sub-cutaneous H460 human lung carcinoma xenografts reduced the amount of tumor growth. Treatment started 14 days after tumor cell inoculation (s.c. shoulder injection of  $10^6$  cells) by injecting MBOs (50  $\mu$ g 2'-O-methyl RNA oligonucleotides per g tumor) into the palpable tumor mass three times per week for two weeks. Vinorelbine (VNB; also referred to as navelbine (NVB) (15 mg/kg i.p.) was injected on days 17 and 24. Tumor size was measured with calipers three times per week. At the end of the treatment period (day 24), the mean relative tumor growth of mice treated with a combination of C5 + G4 AS MBOs and VNB was ~ 70 % reduced compared to those treated with scrambled control MBOs and VNB. Treatments with C5 AS MBO and VNB resulted in a ~ 60 % reduction of tumor size, compared to scrambled control (Fig. 14).

Initial systemic PS-oligonucleotide studies were designed without any chemotherapeutic agents. SCID-RAG2 mice were inoculated with H460 human lung carcinoma cells (s.c. shoulder injection of  $10^6$  cells) and treatments with G4 and F3 AS PS-oligonucleotides, as well as a scrambled control, were initiated three days after tumor inoculation. Nucleobase oligomer injections were administered i.p. at 12.5 mg/kg three times a week for three weeks. At the end of the treatment period, mean tumor sizes in the groups treated with either G4 or F3 AS oligonucleotides were ~ 50 % smaller than in the group treated with a scrambled control oligonucleotides (Fig. 15). The same treatment protocol was tested on female SCID-RAG2 mice inoculated orthotopically with MDA-MB-435/LCC6 human breast carcinoma cells. Two weeks after the last treatment (day 35) tumor volumes of mice treated with F3, C5 or G4 AS oligonucleotides were 70%, 60%, and 45%, respectively, smaller than vehicle controls (Fig. 16).

We conducted additional examination of the antitumor effects of G4 AS oligonucleotides in SCID-RAG2 mice bearing xenografts of H460 human non-small-cell lung tumors implanted subcutaneously. Saline-treated control tumors grew reproducibly to a size of  $0.75 \text{ cm}^3$  within approximately 24 days (Fig. 17). Oligonucleotide treatments were initiated three days after tumor cell inoculation. G4 AS oligonucleotides (5 to 15 mg/kg) were administered using a treatment schedule of i.p. injections given once a day on days 3-7, 10-14, and 17-21. The treatment with 5 or 15 mg/kg G4 AS oligonucleotides greatly delayed tumor growth: on day 24 mean tumor sizes were 0.75, 0.45 and  $0.29 \text{ cm}^3$  in control, 5 and 15 mg/kg treated groups, respectively (Fig. 18A). There was a dose-dependent inhibition of tumor growth. Tumor size in mice treated with 15 mg/kg G4 AS oligonucleotides was significantly smaller than in control groups, and represented 39% of control mean tumor size. In contrast, administration of G4 SC oligonucleotides at 15 mg/kg provided no therapeutic activity (Fig. 17). None of the mice treated with oligonucleotides displayed any signs of toxicities, and both doses of

oligonucleotides were well tolerated. A dose of 15 mg/kg was selected for the future combination treatment regimens with anticancer drugs.

**Example 11: XIAP expression is reduced in H460 tumors treated with G4 AS oligonucleotides**

To correlate the tumor growth inhibitory effects of G4 AS oligonucleotides with XIAP protein expression, we examined the changes in XIAP expression at the end of the *in vivo* treatment with 15 mg/kg of G4 AS and SC oligonucleotides. At day 21 or 24 post-tumor inoculation when tumors reached 1 cm<sup>3</sup> in size (Fig. 17), tumors were harvested and lysates from tumor homogenates were used for western blot analysis. XIAP and  $\beta$ -actin antibodies against the human protein were used, allowing for determination of human XIAP levels obtained from tumor cells specimens without contamination from XIAP derived from mouse cells. XIAP protein levels in tumors treated with G4 AS oligonucleotides were significantly reduced to approximately 85% of control tumors ( $P < 0.005$ ) (Figs. 18A and 18B). Tumors treated with G4 SC oligonucleotides were reduced in size by 24% of control tumors. These results indicated that inhibition of H460 tumor growth by G4 AS oligonucleotides correlated with the down-regulation of XIAP protein expression.

**Example 12: Histopathology of tumor specimens**

To evaluate whether XIAP AS oligonucleotide administration results in direct tumor cell kill, we examined the histology of tumors after treatment both for morphology and ubiquitin immunostaining (Figs. 19A and 19B). At day 21 or 24 post-tumor inoculation, tumors treated with 15 mg/kg of G4 AS oligonucleotides, SC oligonucleotides, or saline control were excised, sectioned, and stained with hematoxylin and eosin. The results demonstrate that tumors in animals administered given XIAP AS

oligonucleotides treatment contained an increased number of dead cells, identified morphologically by their amorphous shape and condensed nuclear material (Fig. 19A).

The degradation of proteins is largely ubiquitin–proteasome-dependent; increased ubiquitin expression has been observed during apoptosis. Thus, we examined the ubiquitin expression in the tumors sections used for hematoxylin and eosin staining. As shown in Fig. 19B, tumors in mice administered XIAP AS oligonucleotides displayed more intense immunohistochemical staining, relative to tumors in control or SC ODN-treated mice. These data indicate that there is more free ubiquitin and/or ubiquitinated-protein in XIAP AS nucleobase oligonucleotide-treated tumor cells than in control tumors.

#### **Example 13: Combined treatment of G4 AS oligonucleotides with vinorelbine**

To evaluate whether combined treatments of G4 AS nucleobase oligomers and vinorelbine (VNB), a chemotherapeutic agent used for lung cancer treatment, may result in any cooperative effects, we compared the therapeutic efficacy of VNB in the presence and absence of G4 AS oligonucleotides or G4 SC oligonucleotides. Treatment regimens were initiated on day 3 after tumor inoculation. Fig. 20A shows the *in vivo* efficacy results for 5 mg/kg and 10 mg/kg doses of VNB given to H460 tumor-bearing mice and compared with saline controls. Each of the two regimens induced significant tumor growth suppression in a dose-dependent manner without showing significant signs of undesirable toxicity (i.e., body weight loss). When administration of G4 AS oligonucleotides (15 mg/kg) was combined with VNB (5 mg/kg) for the treatment of H460 tumors, even more pronounced delay of H460 tumor growth was observed compared to either treatment administered alone (Fig. 20B). Again, the mice did not show any significant signs of toxicity (i.e., body weight loss). The mean tumor sizes in mice treated with 5 mg/kg VNB in the presence or absence of G4 AS or SC

oligonucleotides were compared on day 29 (Figs. 20A and 20B). The average tumor size in the group of VNB and G4 AS oligonucleotides was  $0.22 \pm 0.03 \text{ cm}^3$ , which was significantly smaller than the average tumor size in animals treated with 5 mg/kg VNB alone or with a combination of VNB G4 SC oligonucleotides ( $0.59 \pm 0.04$  and  $0.48 \pm 0.05 \text{ cm}^3$ , respectively).

## Methods

The results obtained in Examples 5-13 were obtained using the following methods.

### Oligonucleotide synthesis

A library of over 96 non-overlapping chimeric, or mixed-backbone (MBO), 19-mer antisense oligonucleotides was synthesized as 2x2 MBO oligonucleotides, composed of two flanking 2'-O-methyl RNA residues at either end with phosphorothioate linkages, and a central core of 15 phosphodiester DNA residues. Each final product was desalted by Sephadex G-25 chromatography (IDT Inc., Coralville, IA). This chimeric wingmer configuration, and mix of phosphorothioate and phosphodiester linkages (referred to as 2x2 PS/PO), provided adequate stability while also reducing non-specific toxicity associated with phosphorothioate residues. Fully phosphorothioated non-chimeric (DNA) antisense oligonucleotides for *in vivo* and *in vitro* studies were synthesized by Trilink Biotech and purified by RP-HPLC.

### Antisense oligonucleotide screening

T24 bladder carcinoma cells, transfected with 1-1.2  $\mu\text{M}$  oligonucleotide-lipofectin complexes for 24-48 hours, were assessed to determine the ability of each oligonucleotide to knock-down XIAP protein. Positive hits were reconfirmed for their ability to knock-down (i) XIAP protein levels at 12-18 hours of transfection by western

analysis, and (ii) XIAP mRNA levels at 6-9 hours of transfection by quantitative RT-PCR (see below) in T24 bladder carcinoma cells and H460 lung carcinoma cells. Candidate oligonucleotides were identified and tested further. Identified 2x2 PS/PO chimeric oligonucleotides showed a dose-dependent ability to decrease XIAP mRNA levels at 6-9 hours in the range of 400-1200 nM concentrations. Exemplary oligonucleotides are shown in Table 5.

Table 5

Oligonucleotide	Sequence*		SEQ ID NO:
F3 AS	<b>ATCTTCTCTTGAAAATAGG</b>	(PS)	278
F3 AS	<u>A</u> UCTTCTCTTGAAAATAG <u>G</u>	(2x2 PS/PO)	279
F3 RP	<b>GGATAAAAGTTCTCTTCTA</b>	(PS)	280
G4 AS	<b>GCTGAGTCTCCATATTGCC</b>	(PS)	281
G4 AS	<u>G</u> CTGAGTCTCCATATTG <u>C</u>	(2x2 PS/PO)	282
G4 SC	<b>GGCTCTTTGCCCACTGAAT</b>	(PS)	283
C5 AS	<b>ACCATTCTGGATAACCAGAA</b>	(PS)	284
C5 AS	<u>A</u> CCATTCTGGATAACCAG <u>AA</u>	(2x2 PS/PO)	285
C5 RP	<b>AAGACCATAGGTCTTACCA</b>	(PS)	286
AB6 AS	<b>GGGTTCCTCGGGTATATGG</b>	(PS)	287
AB6 RP	<b>GGTATATGGCGTCCTTGGG</b>	(PS)	288
DE4 AS	<b>GGTATCTCCTTCACCAGTA</b>	(PS)	289
DE4 RP	<b>ATGACCACTTCCTCTATGG</b>	(PS)	290
D7 AS	<b>GATTCACCTCGAATATTAA</b>	(PS)	291
D7 RP	<b>AATTATAACGTTCACTTAG</b>	(PS)	292

\*Bold residues= DNA residues with phosphorothioate linkages, underlined residues= 2'-O-methyl RNA bases, plain type= phosphodiester DNA residues.



**Tumor cell line and animal xenografts model**

The human non-small cell lung cancer cell line (large cell type) NCI-H460 (H460) was obtained from ATCC and maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were used in exponential growth phase, up to a maximum of 25 *in vitro* passages. Male SCID-RAG2 mice (7-9 weeks old, 23-26g) were obtained from British Columbia Cancer Agency Joint Animal Facility breeding colony and kept in aseptic environments. A tumor model of NCI-H460 cells in SCID-RAG2 mice was established by subcutaneous implantation of 1x10<sup>6</sup> NCI-H460 cells on the back of mice.

**Treatment of cells with antisense and anticancer drugs**

One day prior to transfection, H460 cells were plated in 6- or 96-well tissue culture plates. Phosphorothioate antisense oligonucleotides were delivered into cells with Lipofectamine 2000 (Life Technologies) in the form of liposome-oligonucleotide complexes. Following a 4.5 or 6 h transfection, the transfection medium was replaced with RPMI medium containing 10% FBS, and the cells incubated for another 24 or 48 h.

**Real-time quantitative RT-PCR**

Total RNA from H460 cells treated with liposome-oligonucleotide complexes for 6 hours was immediately isolated using RNeasy mini spin columns and DNase treatment (QIAGEN, Valencia, CA). Specific XIAP mRNA was measured using a real-time quantitative RT-PCR method. XIAP forward and reverse primers (600 nM) and probe (200 nM) (5'-GGTGATAAAGTAAAGTGCTTTCACTGT-3' (SEQ ID NO 293), 6FAM- CAACATGCTAAATGGTTCCAGGGTGCAAATATC-TAMRA (SEQ ID NO: 294), and 5'-TCAGTAGTTCTTACCAGACACTCCTCAA-3' (SEQ ID NO: 295) were designed to span exon 3-4 and 4-5 junctions. One of the primers, as well as the probe,

was designed to overlap an intron-exon boundary to block detection of any possible genomic DNA contamination. The RNA was reverse-transcribed and PCR amplified using the TaqMan EZ RT-PCR kit (PE/ABI, Foster City, CA) in the ABI prism 7700 Sequence Detection System (PE/ABI). The thermal cycling condition for the RT step were 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min, followed by 45 cycles of PCR (at 94°C for 20 s and 60°C for 1 min per cycle). The XIAP mRNA level of each sample was calculated relative to untreated control cells. XIAP mRNA levels were determined by the cycle threshold method (Ct) using a threshold of 30X the baseline SD, and XIAP levels were normalized for GAPDH content, using PE/ABI supplied primers and probe.

#### **Western blot analysis**

The cells or tumor tissue samples were lysed with ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 0.02% sodium azide) containing protease inhibitors (Complete-Mini protease inhibitor tablets; Boehringer Mannheim GmbH, Mannheim, Germany). After incubation for 30 min on ice, samples were centrifuged at 10,000 rpm for 15 min, and stored at -20°C. Protein content in the lysed extracts was determined using a detergent-compatible Bio-Rad assay (Bio-Rad Labs, Hercules, CA). Equal amounts of protein (40 µg/lane) were separated on 12% SDS-polyacrylamide gels or 4-15% gradient SDS-polyacrylamide pre-made gels (Bio-Rad) and transferred to nitrocellulose membranes. Primary antibodies against XIAP, Bcl-2 (DAKO, Glostrup, Denmark), Bax (Sigma, St. Louis, MO),  $\beta$ -actin (Sigma), caspase-3 (BD PharMingen, San Diego, CA), and PARP (BD PharMingen) were used. The secondary antibody was the appropriate horseradish-conjugated anti-mouse or anti-rabbit IgG (Promega, Madison, WI). Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, England)

and visualized after exposure to Kodak autoradiography film. Scanning densitometry (Molecular Dynamics, Sunnyvale, CA) was performed to quantify band intensities by volume/area integration. The amount of XIAP, caspase-3, Bcl-2 and Bax in cells was normalized to their respective lane  $\beta$ -actin levels, upon stripping and reprobing.

### **Measurement of cell growth and viability or death**

Growth inhibition of H640 cells was determined by the colorimetric MTT cell viability/proliferation assay. In brief, cells were treated with liposome-oligonucleotide complexes for 4.5 h, then incubated for another 48 h at 37°C in medium without transfection reagent or oligonucleotides in the presence or absence of anticancer drugs. MTT (25  $\mu$ g/well) was added to each well, and the plates incubated for 3 h at 37°C. Following the incubation step, the colored formazan product was dissolved by the addition of 200  $\mu$ l DMSO. Plates were read using the microtiter plate reader (Dynex Technologies Inc., Chantilly, VA) at a wavelength of 570 nm. The percentage of surviving cells in wells treated with oligonucleotides was normalized to untreated controls. All assays were performed in triplicate.

### **Apoptotic flow cytometric assays**

Cells were treated with liposome-oligonucleotide complexes for 4.5 h, and incubated for another 48 h in the medium without transfection reagent at 37°C. Following incubation, cells were harvested, washed twice with sample buffer (0.5% glucose in PBS without  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ), and fixed in cold 70% ethanol at 4°C for at least 18 hrs. Samples were centrifuged at 3000 rpm for 10 min, then resuspended in sample buffer containing 50  $\mu$ g/ml propidium iodide (PI) and 400 U/ml RNase A. Samples were incubated for 30 min at room temperature and 30 min on ice, followed by flow cytometry analysis. EXPO Software (Applied Cytometry Systems, Sacramento, CA) was used to

generate histograms, which were used to determine the cell cycle phase distribution after debris exclusion. The Sub G1/G0 cell fraction was considered as representative for apoptotic cells.

### **Nuclear morphology**

Cells were treated with liposome-oligonucleotide complexes for 4.5 h, and incubated for another 48 h at 37°C in the medium without transfection reagent or oligonucleotides. Cells were harvested and stained with 0.10 µg/ml DAPI (4',6-diamidino-2-2-phenylindole) for 30 min at room temperature. Cells were placed on a glass slide, cytopun, and viewed with a Leica microscope and 40X objective lens under UV fluorescent illumination. Digital images were captured using Imagedatabase V. 4.01 Software (Leica, Germany).

### ***In vivo* antitumor activity**

Efficacy experiments were conducted in male RAG2 immunodeficient mice bearing NCI-H460 tumours or female RAG2 mice bearing LCC6 tumors. Treatments were commenced on day 3 after tumor inoculation. Saline (controls), G4 AS oligonucleotides (5 or 15 mg/kg), or G4 SC oligonucleotides (5 or 15 mg/kg) were administered i.p. daily for five doses a week over a three week regimen. Vinorelbine (VNB, 5 or 10 mg/kg) was administered i.v. via the tail vein, either alone or in combination with oligonucleotides, at day 3, 7, 11 and 17 after tumor inoculation. When oligonucleotides were administered in combination with VNB, the drug treatment was performed four hours after ODN treatment.

Mice were observed daily. Body weight measurements and signs of stress (e.g., lethargy, ruffled coat, ataxia) were used to detect possible toxicities. Animals with ulcerated tumor, or tumor volumes of 1 cm<sup>3</sup> or greater were killed. Digital caliper

measurements of tumors were converted into mean tumor size (cm<sup>3</sup>) using the formula:  $\frac{1}{2}[\text{length (cm)}] \times [\text{width (cm)}]^2$ . An average tumor size per mouse was used to calculate the group mean tumor size  $\pm$  SE (n = 6 mice) from at least two independent experiments per group.

### **Tumor and tissue processing**

Mouse tumors were collected on day 21 or 24 post-tumor inoculation and treatment. One portion of the tumor tissue was fixed in formalin. Paraffin-embedded tissues were sectioned and subjected to gross histopathology using hematoxylin and eosin staining and immunohistochemistry for ubiquitin expression. The other portion of the tumor was homogenized in lysis buffer for western blot analysis (see above).

### **Statistical analyses**

Student's t test was used to measure statistical significance between two treatment groups. Multiple comparisons were done using one-way ANOVA and a post-hoc test that compared different treatment groups by the Shellé test criteria (Statistica release 4.5, StatSoft Inc., Tulsa, OK). Data were considered significant for a *P*-value of <0.05.

### **Example 14: Antisense HIAP1 oligonucleotides decrease HIAP1 RNA and polypeptide expression**

A library of 15 HIAP1 antisense nucleobase oligomers as oligonucleotides was screened for protein knock-down by western blot analysis and for RNA knock-down by TaqMan, using the primers and probes described in Example 3, above, under two different conditions. HIAP1 RNA levels may be detected using standard Northern blot analyses or RT-PCR techniques. The antisense oligonucleotides were administered to cells under basal conditions or under cycloheximide-induction conditions (24 hour

treatment with sub-toxic doses). Cycloheximide (CHX) can lead to a 10- to 200-fold induction of HIAP1 mRNA depending on the cell line treated. This in turn leads to an increase in HIAP1 protein, as seen on a Western blot (70 kDa band). This effect of CHX is via two distinct mechanisms of action. First, CHX activates NFkB, a known transcriptional inducer of HIAP1, by blocking the *de novo* synthesis of a labile protein, Ikb, which is an inhibitor of NFkB. This effect is mimicked by puromycin, another protein synthesis inhibitor, and by TNF-alpha, which induces a signaling cascade leading to the phosphorylation, ubiquitination, and degradation of Ikb. Only CHX leads to a further stabilization of the HIAP1 mRNA, as seen by the decreased rate of disappearance of HIAP1 message in the presence of actinomycin D, to block *de novo* transcription, and CHX, as opposed to actinomycin D and puromycin or TNF-alpha combined.

SF295 glioblastoma cells were transfected with lipofectin and oligonucleotide (scrambled survivin, no oligonucleotide, antisense APO 1 to APO 15) or left untreated. RNA was isolated from the cells six hours after transfection and the level of HIAP1 mRNA was measured by quantitative PCR (TaqMan analysis), normalized for GAPDH mRNA, with the value for the scrambled survivin oligonucleotide transfection set as 1.0. The results of this experiment, a compilation of three separate experiments, are shown in Fig. 21. The scrambled survivin oligonucleotide, the mock transfection, and untreated (non-transfected) cells, all showed similar HIAP1 mRNA levels. Of the 15 antisense oligonucleotides, seven (APO 1, 2, 7, 8, 9, 12, 15) showed an almost 50% decrease when compared to mock transfection or survivin scrambled control oligonucleotide transfection (5'-mUmAmAGCTGTTCTATGTGmUmUmC-3'; SEQ ID NO: 296) (Fig. 21). Some of the oligonucleotides led to an induction in HIAP1 mRNA, which may be a stress response to a non-specific toxic oligonucleotide. An antisense oligonucleotide may still be effective at knocking down HIAP1 protein levels even if the message is increased if the oligonucleotide is able to interfere with the translation process.

The effect of HIAP1 antisense nucleobase oligomers on HIAP1 protein and mRNA expression was also examined in cells induced to express HIAP1. SF295 cells were transfected with oligonucleotides, or were mock transfected. The transfected cells were then treated with 10 µg/ml cycloheximide for 24 hours to induce 70 kDa HIAP1 mRNA and protein. Protein levels were measured by western blot analysis with an anti-HIAP1 polyclonal antibody, and normalized against actin protein in a re-probing of the same blots. Scans of the western blot results are shown in Fig. 22A. The densitometric scan results were plotted against the mock results (set at 100%) in Fig. 22B. A line is drawn at 50% to easily identify the most effective antisense oligonucleotides. The transfection process itself (e.g., mock or scrambled survivin) induces HIAP1 protein compared to the untreated sample as shown on the western immunoblot.

Of the 15 tested nucleobase oligomers, six of them (APO 1, 2, 7, 8, 12, and 15) showed high activity, or had significant activity in both the protein and mRNA assays, and did not cause a stress-induced increase in HIAP1 mRNA, such as that seen with APO 4, 6, 11, 13, 14 (Fig. 21), and by control oligonucleotides to APO 2 (mismatch or reverse polarity, see text below and Figs. 23 and 24). Note that APO 6 also showed evidence of toxicity as seen by the general decrease in total protein (Fig. 23).

To further investigate the efficacy of HIAP1 antisense oligonucleotides under cycloheximide induction conditions, changes in HIAP1 mRNA were measured by TaqMan real time PCR 6 hours after transfection with APO 2, which targets an Alu repeat within an intron of HIAP1 and results in the greatest block of CHX-induced upregulation of HIAP1 mRNA and protein. Controls for this experiment were three oligonucleotides for APO 2: one scrambled sequence (same base composition but random order, 5'-AAGGGCGGCGGAGTGAGAC-3'; SEQ ID NO: 297), one reverse polarity (same base composition, same sequential order but in the opposite direction, 5'-AGAGG

ACGGAGTCGGAGGC-3'; SEQ ID NO: 298), and one mismatch sequence (containing four base mismatches out of 19 bases, 5'-CGGAGCGTGAGGATGGAGA-3'; SEQ ID NO: 299).

Transfection of the APO 2 antisense into cells resulted in a 50% decrease in mRNA compared to a scrambled survivin control and matched perfectly with the protein results, while the scrambled control for APO 2 (H1 sc apo 2 in Fig. 24) did not change HIAP1 mRNA levels at all (repeated twice here, and in two different experiments). However, the mismatch control ODN (H1 mm apo 2) and the reverse polarity control oligonucleotide (H1 RV apo 2) showed an induction of 6 to 7 fold in HIAP1 mRNA at 6 hours. These oligonucleotides no longer targeted HIAP1, as expected, but may still target Alu repeats because of the degeneracy and repeat nature of these sequences. Therefore, it is possible that these two controls are toxic to the cell and cause a stress response that leads to the induction of HIAP1. This effect may also occur with the antisense APO 2 oligonucleotide, but in this case, APO 2 also causes the degradation of the induced HIAP1 mRNA which results in a relative decrease of HIAP1 mRNA, compared to a scrambled survivin control, as well as decreasing the relative fold induction of HIAP1 protein after transfection and CHX treatment, compared to scrambled survivin control oligonucleotide.

The six antisense HIAP1 nucleobase oligomers include two very effective oligonucleotides against an intronic sequence (APO 1, and APO 2, with APO 2 demonstrating the better activity). These oligonucleotides could be used therapeutically for treatment of cancer or autoimmune disorders. The oligonucleotides against an intronic sequence would likely only target pre-mRNA (very short-lived target) and not the mature, processed form of HIAP1. Typically, introns are not targeted for antisense except when one wants to alter splicing by targeting the intron-exon boundaries or the branching point. These usually result in the skipping of an exon rather than RNase-



mediated degradation of the message. Both mechanisms would likely be favorable for the enhancement of apoptosis, as the skipping would result in the loss of the exon encoding the first two important BIR domains of HIAP1. The APO 2 antisense ODN also targets an intron of survivin for 18 consecutive bases out of 19, but we did not see any loss of survivin protein; only HIAP1 was decreased after the oligo treatment, demonstrating the specificity of the HIAP1 antisense oligonucleotide. These antisense oligonucleotides hit Alu sequences in the HIAP1 intron and potentially in many other genes, and induce the cancer cells to die (see below), which may be as a result of down regulating HIAP1 and some other critical genes, and thus of therapeutic value if it is not too toxic to normal cells.

Cancer cells have reportedly more Alu-containing transcripts and may therefore be more sensitive to apoptosis induction with an Alu targeting nucleobase oligomer. Furthermore, this killing effect of nucleobase oligomers APO 1 and APO 2 may be due to the combined effect of both targeting Alu sequences and HIAP1 simultaneously. This dual effect would result in an effective way to prevent the normal stress response of HIAP1 induction through the NFkB pathway, when the cell is exposed to certain toxic agents. This stress response is most likely part of the cancer cell's anti-apoptotic program. By blocking HIAP1 expression, we counter this anti-apoptotic stress response and precipitate the cancer cell's demise.

#### **Example 15: HIAP1 antisense oligonucleotides increase cytotoxicity and chemosensitization**

The effect of HIAP1 antisense nucleobase oligomers on the chemosensitization of SF295 cells was also evaluated. Cells were transfected with one of three different antisense oligonucleotides (APO 7, APO 15, and SC APO 2 (control)). Twenty-four hours after transfection with the oligonucleotides, the cells were incubated with

adriamycin for an additional 24 hours before assaying by for cell survival by assaying WST-1.

The WST-1 survival curves for SF295 cells transfected with the above-described HIAP1 oligonucleotides and then treated with increasing concentrations of adriamycin are shown in Fig. 25. The two oligonucleotides that resulted in a decrease in HIAP1 mRNA also showed a decrease in survival when treated with adriamycin compared to cells treated with an oligonucleotide that did not reduce HIAP1 mRNA levels. Therefore, reducing HIAP1 levels by antisense, or other means, can chemosensitize a glioblastoma cell line that is highly resistant to the cytotoxic action of many chemotherapeutic drugs.

An additional 89 HIAP1 antisense sequences that can be employed in the methods of the invention are shown in Table 6. Sequences that are 100% identical between human HIAP1 and human HIAP2, or have one or two mismatches, are in bold.

Table 6

Nucleobase oligomer sequence	SEQ ID NO:	Nucleobase oligomer sequence	SEQ ID NO:	Nucleobase oligomer sequence	SEQ ID NO:
AGCAAGGACAAAGCCAGTC	300	TTAAGCCATCAGGATGCCA	332	GATTTTCTCTGAACTGTC	364
TGTAAACCTGCTGCCAGA	301	GCTACAGAGTAAGCTGTGT	333	CTATAATCTCTCCAGTTG	365
AGAAGTCGTTTCTCTCCT	302	CTCTAGGAGGTAGTTTGT	334	ACACAAGATCATGACTAG	366
CCGAGATTAGACTAAGTCC	303	AAGAAAAGGGACTAGCCTT	335	TCTGCATTGAGTAAGTCTA	367
ACTTTCTCTTTAATTCAC	304	CAGTTCACATGACAAGTCG	336	TCTTTTCTCTCAGTTGCTC	368
TCCAAAACACAGGTACTAT	305	GACTCCTTCTGAGACAGG	337	GTGCCATTCTAATCTTCCG	369
CATTCTCAGCGGTAAACAG	306	ATTACACACAGTGTAAATAG	338	GTAGACTATCCAGGATTGG	370
ACATCATCTCTCATCTCTCA	307	CAGAAGCAATTTGACCTTGT	339	AGTTCTCTTGGTGTAAAG	371
AATGTAAACCTTCAACCATC	308	CCAGCATCAGGCCACAACA	340	TGGTATCAATCAGTTCTCT	372
TTTGTATTTCATCACTGTC	309	TTTCAGTAGGACTGTCTCC	341	GCAGAGAGTTTCTTGAATAC	373
TCACATCTCATTTACCAAC	310	TGCAGCTAGGATACAACTT	342	ATGTCCTGTTGCACAAATA	374
CCAGGTGGCAGGAGAAACA	311	AGAGGTAGCTTCCAAGTTG	343	CTGAAACATCTTCTGTGGG	375
TGCAGACTTCAATGCTTTG	312	GAAGTAAATGAGTGTGTGGA	344	TTTCTTCTTGTAGTCTCCG	376
TAAGCAAGTCACCTGTGGCT	313	GGATTTGATGGAGAGTTTG	345	CTTCTTTGTCCATACACAC	377
CTGAGTCGATAATACTAGC	314	GAACTTCTCATCAAGGCAG	346	GGAATAAACACTATATGGACA	378
ACTAGCCATTAGTAAGAG	315	AGTCTCTATGTAGTAAAG	347	CATACACTAGATGACCAC	379
CAACAGCAGAGACCTTGTCTC	316	CAATTTTCCACCACAGGCA	348	TGTACCTTGAATTTGACTC	380
ATAGCATACCTTGACCAG	317	CATTATCCTTCGGTTCCCA	349	GAAATGTACGAACTGTACC	381
CATCTGTAGGCTAAGATGG	318	CTCAGGTGTTCTGACATAG	350	GATGTTTTGGTCTTCTTTC	382
AGTTACCAGATGCCATCTG	319	GCTCAGATTAGAAACTGTG	351	CTATCATTTCTCTTAGTTTC	383
AATCTACTCTGATAGTGG	320	CTGCAATGTTCTGCAATGCT	352	ACACCTGGCTTCATGTTCC	384
GTTTCTGAAGCCCAACATCA	321	TTAACTAGAACACTAGAGG	353	GACTACAGGCACATACCAC	385
TCAACTTATCACCTCCTGA	322	CATAATAAAAACCCGCACT	354	TGCCTCAGCCTGGGACTAC	386
AAGAACTAAACATTGTAGAG	323	CACCATCACAGCAAAAAGCA	355	AGGATGGATTCAAACTCCT	387
GTAGACAACAGGTGCTGCA	324	CTCCAGATTCCCAACACCT	356	GAGAAAATGTCCCTGGTG	388
ATGTCCTCTGTAAATTATGG	325	GGAAACCACTTGGCATGTT	357	GCCACAACAGAAAGCATTTG	389
TACTTGGCTAGAACATGGA	326	GTTCAAGTAGATGAGGGTA	358		
GAAGCAACTCAATGTTAAG	327	GATAATTGATGACTCTGCA	359		
TTTGGTCTTTTGGACTCAG	328	ATGGTCTTCTCCAGGTTCA	360		
CCATAGATCATCAGGAATA	329	GCATTAAATCACAGGGGTAT	361		
CAGGACTGGCTAACACATC	330	TAAAGCCCATTTCCACGGC	362		
TTTAAATGGCAGGCATCTCC	331	TGTTTTACAGGCTTCTAC	363		

We also analyzed human HIAP2 for sequences suitable for use as antisense nucleobase oligomers. Identified sequences are shown in Table 7.

**Table 7**

<b>Nucleobase oligomer sequence</b>	<b>SEQ ID NO:</b>	<b>Nucleobase oligomer sequence</b>	<b>SEQ ID NO:</b>
TTCTGAAAACTCTTCAATG	390	CTCAGAGTTTCTAGAGAAT	426
CTTAGCATAAAGTATCAGT	391	ATGTTCTCATTCGAGCTGC	427
CAAAAAAGTACTGCTTAGC	392	TGAACTGGAACACTAGATG	428
CAAGATAAACTTGTCTT	393	GCTCAGGCTGAACTGGAAC	429
TATCAGTCATGTTGTAAAC	394	TTGACATCATCATTGCGAC	430
CTAAATAACCTGTTTCATCA	395	ACCATCACAAACAAAGCAT	431
AGCACACTTTTTTACACTGC	396	CCACTTGGCATGTTCTACC	432
ACCACTATTATTCTTGATC	397	TCGTATCAAGAACTCACAC	433
TGTATTTGTTTCCATTTCC	398	GGTATCTGAAGTTGACAAC	434
ACTGTAAACTCTATCTTTG	399	TTTCTTCTCCAGTGGTATC	435
CTTAAGTGGGCTAAATTAC	400	TTCTCCAGGTCCAAAATGA	436
CCTTCATATGGTCACACTA	401	ACAGCATCTTCTGAAGAAC	437
GGTTACAAGCTATGAAGCC	402	CACAGGTGTATTTCATCATG	438
CTAAGCAACTATAGAATAC	403	CCAGGTCTCTATTAAAGCC	439
TCCTTGATTTTTTCACAGAG	404	TTCTCTCCAGTTGTCAGGA	440
ATACTAACTTAAAGCCCTG	405	GAAGTGCTGACACAATATC	441
GGGTGTAGTAACTCTTTC	406	TTTTCTTCTCCTCCTCTC	442
TAGAACACAACCTTTGGG	407	CATCTGATGCCATTTCTTC	443
CTCTGAATTTCCAAGATAC	408	AGCCATTCTGTTCTTCCGA	444
TTTACTGGATTTTATCTCAG	409	CCAGGATAGGAAGCACACA	445
TGAGTAGGTGACATGCTG	410	ATGGTATCAATCAGTTCTC	446
GGAGGCAGTTTTTGTGCATG	411	CCGCAGCATTTCTTTAAC	447
CTATCTTCCATTATACTCT	412	CAGTTTTTGAAGATGTTGG	448
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TCCTTTCTGAGACAGGCAC	414	GGGCATTTTCTTAGAGAAG	450
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ACCTTGTCATTACACCAG	416	GAAATGTACGAACAGTACC	452
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GCTTTTGAATAGGACTGTC	418	CCATCTTTTCAGAAACAAG	454
GAGATGTCTTCAACTGCTC	419	CTATAATTCTCTCCAGTTG	455
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GCTCTTGCCAATTCTGATG	422	GTAACCTCCTGAAATGATGC	458
ACCCTATCTCCAGGTCCTA	423	CAACAAATCCAGTAACTCC	459
ACAGGCAAAGCAGGCTACC	424	CACCATAACTCTGATGAAC	460
GTTCTGACATAGCATCATC	425		

**Other Embodiments**

All publications and patent applications mentioned in this specification, including U.S. Patent Nos. 5,919,912, 6,156,535, and 6,133,437, are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed is:

### Claims

1. A substantially pure nucleobase oligomer of up to 30 nucleobases in length, said nucleobase oligomer comprising at least eight consecutive nucleobases of a sequence selected from the group consisting of SEQ ID NOs: 97, 98, 99, 143, 147, 151, 287, 289, and 300-460.

2. The nucleobase oligomer of claim 1, wherein said nucleobase oligomer consists essentially of a sequence selected from the group consisting of SEQ ID NOs: 97, 98, 99, 143, 147, 151, 287, 289, and 300-460.

3. The nucleobase oligomer of claim 2, wherein said nucleobase oligomer consists of a sequence selected from the group consisting of SEQ ID NOs: 97, 98, 99, 143, 147, 151, 287, 289, and 300-460.

4. The nucleobase oligomer of any one of claims 1-3, wherein said nucleobase oligomer is an oligonucleotide.

5. The nucleobase oligomer of any one of claims 1-4, wherein said oligonucleotide comprises at least one modified linkage.

6. The nucleobase oligomer of claim 5, wherein said modified linkage is selected from the group consisting of phosphorothioate, methylphosphonate, phosphotriester, phosphorodithioate, and phosphoselenate linkages.

7. The nucleobase oligomer of any one of claims 1-6, wherein said nucleobase oligomer comprises at least one modified sugar moiety.

8. The nucleobase oligomer of claim 7, wherein said modified sugar moiety is a 2'-O-methyl group or a 2'-O-methoxyethyl group.

9. The nucleobase oligomer of any one of claims 1-8, wherein said nucleobase oligomer comprises at least one modified nucleobase.

10. The nucleobase oligomer of claim 9 wherein said modified nucleobase is 5-methyl cytosine.

11. The nucleobase oligomer of any one of claims 1-10, wherein said nucleobase oligomer is a chimeric nucleobase oligomer.

12. The nucleobase oligomer of claim 11, wherein said nucleobase oligomer comprises DNA residues linked together by phosphorothioate linkages, said DNA residues flanked on each side by at least one 2'-O-methyl or 2'-O-methoxyethyl RNA residue.

13. The nucleobase oligomer of claim 12, wherein said DNA residues are flanked on each side by at least three 2'-O-methyl or 2'-O-methoxyethyl RNA residues.

14. The nucleobase oligomer of claim 13, wherein said DNA residues are flanked on each side by four 2'-O-methyl or 2'-O-methoxyethyl RNA residues.

15. The nucleobase oligomer of any one of claims 12-14, wherein said RNA residues are linked together by phosphorothioate linkages, and said RNA residues are linked to said DNA residues by phosphorothioate linkages.

16. The nucleobase oligomer of claim 11, wherein said nucleobase oligomer comprises DNA residues linked together by phosphodiester linkages, said DNA residues flanked on each side by at least two 2'-O-methyl or 2'-O-methoxyethyl RNA residues linked together by phosphorothioate linkages.

17. The nucleobase oligomer of claim 16, wherein said DNA residues are flanked on each side by at least three 2'-O-methyl or 2'-O-methoxyethyl RNA residues.

18. A nucleobase oligomer comprising eleven DNA residues flanked on each side by four 2'-O-methyl RNA residues, said nucleobase oligomer consisting of one of the following sequences:

5'-AUUGGTTCCAATGTGUUCU-3' (SEQ ID NO: 155);

5'-ACACGACCGCTAAGAAACA-3' (SEQ ID NO: 16);

5'-ACAGGACTACCACTTGGAA-3' (SEQ ID NO: 157);

5'-UGCCAGTGTGTGATGCUGAA-3' (SEQ ID NO: 27);

5'-GCUGAGTCTCCATATUGCC-3' (SEQ ID NO: 141);

5'-UCGGGTATATGGTGTCTUGA-3' (SEQ ID NO: 41);

5'-AAGCACTGCACTTGGUCAC-3' (SEQ ID NO: 47);

5'-CCGGCCCCAAAACAAAGAAG-3' (SEQ ID NO: 51);

5'-ACCCTGGATACCATTUAGC-3' (SEQ ID NO: 63);

5'-UGUCAGTACATGTTGGCUC-3' (SEQ ID NO: 161); and

5'-UGCACCCTGGATACCAUUU-3' (SEQ ID NO: 151), said residues

linked together by phosphorothioate linkages.

19. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 155.

20. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 16.

21. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 157.

22. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 27.

23. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 141.



24. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 41.

25. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 47.

26. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 51.

27. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 63.

28. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 161.

29. The nucleobase oligomer of claim 18, wherein said nucleobase oligomer consists of the sequence of SEQ ID NO: 151.

30. The nucleobase oligomer of any one of claims 1-29, wherein said nucleobase oligomer inhibits the expression of an IAP in said cell.

31. A method of enhancing apoptosis of a cell in an animal, said method comprising administering to said animal a nucleobase oligomer of any one of claims 1-29 under conditions such that said nucleobase oligomer inhibits the expression of an IAP in said cell.

32. The method of claim 31, further comprising administering to said animal a chemotherapeutic agent.

33. The method of claim 32, wherein said chemotherapeutic agent is a cytostatic agent.

34. The method of claim 32, wherein said chemotherapeutic agent is a cytotoxic agent.

35. The method of any one of claims 31-34, further comprising administering to said animal a chemosensitizer.

36. The method of any one of claims 31-35, further comprising administering to said animal a biological response modifying agent.

37. The method of any one of claims 31-36, wherein said nucleobase oligomer is administered to said animal intravenously or intratumorally.

38. The method of any one of claims 31-37, wherein said animal is a human.

39. A method of treating an animal having a cancer or lymphoproliferative disorder, said method comprising administering to said animal a therapeutically effective amount of a nucleobase oligomer of any one of claims 1-30.

40. The method of claim 39, wherein the cancer is acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, myelodysplastic syndrome, chronic lymphocytic leukemia, polycythemia vera, lymphoma, Hodgkin's disease, Waldenstrom's macroglobulinemia, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma,

carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, schwannoma, meningioma, melanoma, neuroblastoma, or retinoblastoma.

41. The method of claim 39 or 40, further comprising administering to said animal a chemotherapeutic agent.

42. The method of claim 41, wherein said nucleobase oligomer and said chemotherapeutic agent are administered to said animal within five days of each other.

43. The method of claim 42, wherein said nucleobase oligomer and said chemotherapeutic agent are administered to said animal within twenty-four hours of each other.

44. The method of any one of claims 41-43, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, vinorelbine, etoposide, taxol, and cisplatin.

45. The method of claim 41, wherein said chemotherapeutic agent is a cytostatic agent.

46. The method of claim 41, wherein said chemotherapeutic agent is a cytotoxic agent.

47. The method of any one of claims 39-46, further comprising administering to said animal a chemosensitizer.

48. The method of any one of claims 39-47, further comprising administering to said animal a biological response modifying agent.

49. The method of any one of claims 39-48, wherein said nucleobase oligomer is administered to said animal intravenously or intratumorally.

50. The method of any one of claims 39-49, wherein said animal is a human.

51. A pharmaceutical composition comprising a nucleobase oligomer of any one of claims 1-30 and a pharmaceutically acceptable carrier.

52. The pharmaceutical composition of claim 51, further comprising a colloidal dispersion system.

53. A catalytic RNA molecule capable of cleaving XIAP, HIAP1, or HIAP2 mRNA.

54. The catalytic RNA molecule of claim 53, the binding arms of which contain at least eight consecutive nucleobases corresponding to a sequence of any one of Tables 1, 2, 6, and 7.

55. The catalytic RNA molecule of claim 53 or 54, wherein said RNA molecule is in a hammerhead motif.

56. The catalytic RNA molecule of claim 53 or 54, wherein said RNA molecule is in a hairpin, hepatitis delta virus, group 1 intron, VS RNA or RNaseP RNA motif.

57. An expression vector comprising a nucleic acid encoding one or more catalytic RNA molecules of any one of claims 53-56 positioned for expression in a mammalian cell.

58. A method of treating an animal having a cancer or lymphoproliferative disorder, said method comprising administering to said animal an effective amount of a catalytic RNA molecule of any one of claims 53-56.

59. A double-stranded RNA molecule consisting of between 21 and 29 nucleobases, said RNA molecule comprising at least eight consecutive nucleobases corresponding to a sequence of any one of Tables 1, 2, 6, and 7.

60. A double-stranded hairpin RNA molecule consisting of between 50 and 70 nucleobases, said RNA molecule comprising a first domain of between 21 and 29 nucleobases that comprise least eight consecutive nucleobases corresponding to a sequence of any one of Tables 1, 2, 6, and 7; a second domain complementary to said first domain, and a loop domain situated between said first and said second domains such that said first domain and said second domain are capable of duplexing to form said double-stranded hairpin RNA molecule.

61. An expression vector comprising a nucleic acid molecule encoding the double stranded RNA molecule of claim 60 positioned for expression in a mammalian cell.

62. A method of treating an animal having a cancer or lymphoproliferative disorder, said method comprising administering to said animal an effective amount of a double-stranded RNA molecule of claim 59 or 60.

63. An oligonucleotide consisting of a sequence selected from SEQ ID NOs: 97, 98, 99, 143, 147, 151, 287, 289, and 300-460.

Figure 1A

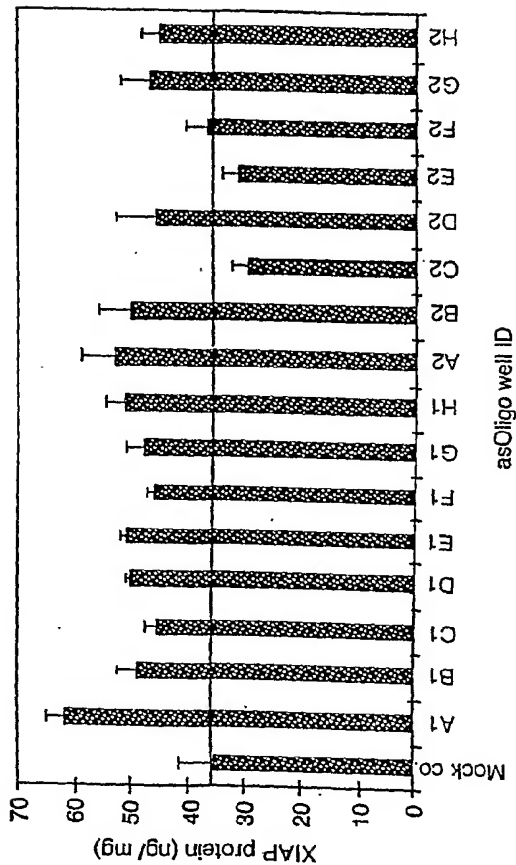


Figure 1B

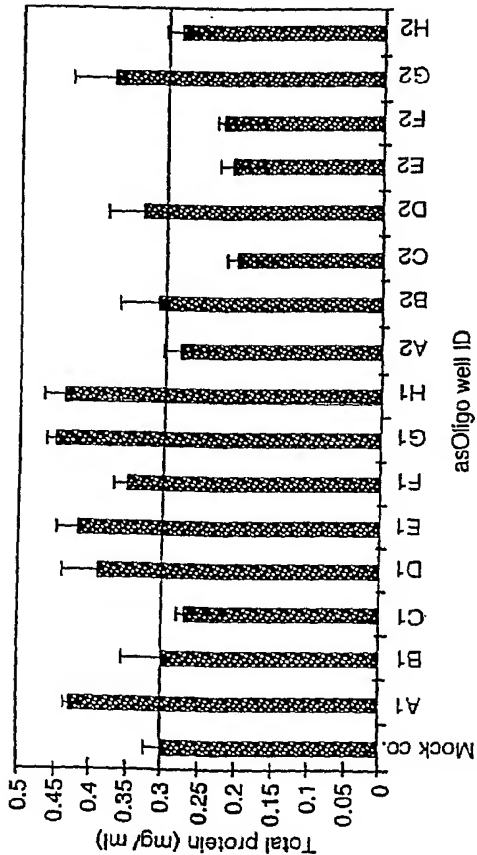


Figure 1C

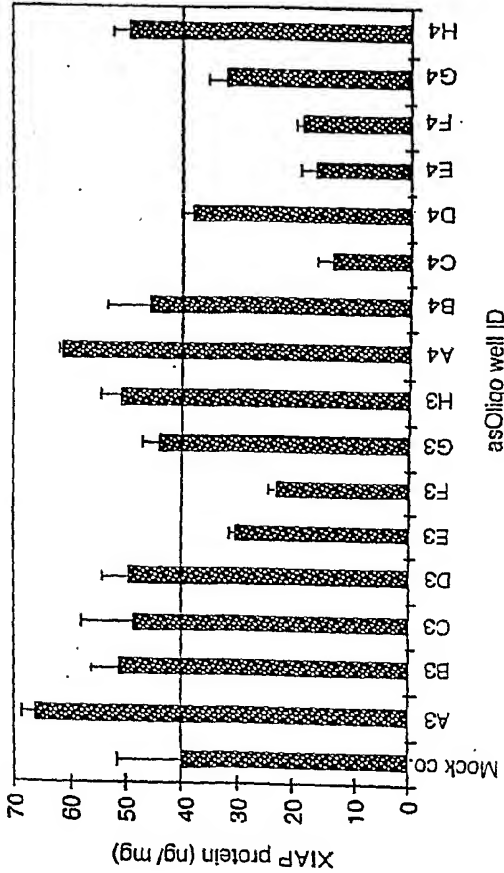


Figure 1D

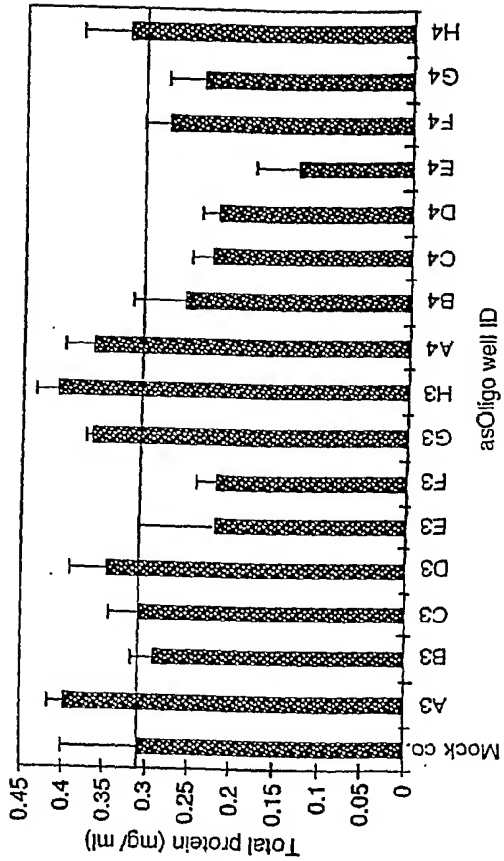


Figure 1E

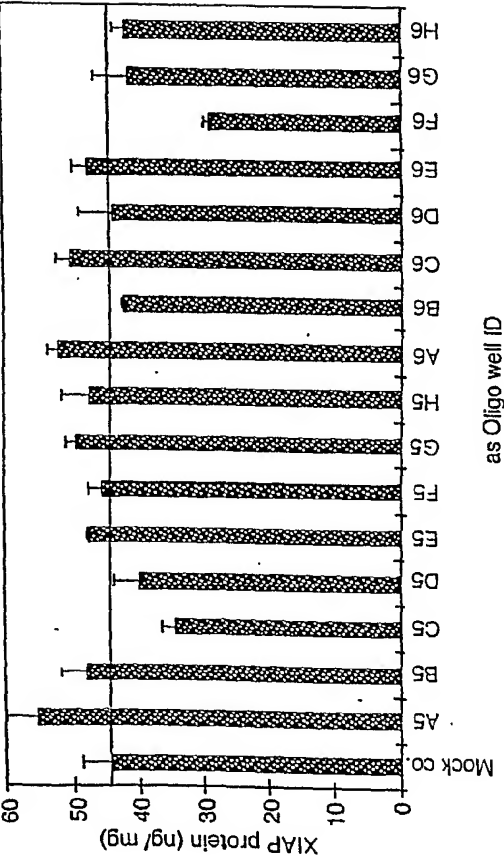


Figure 1F

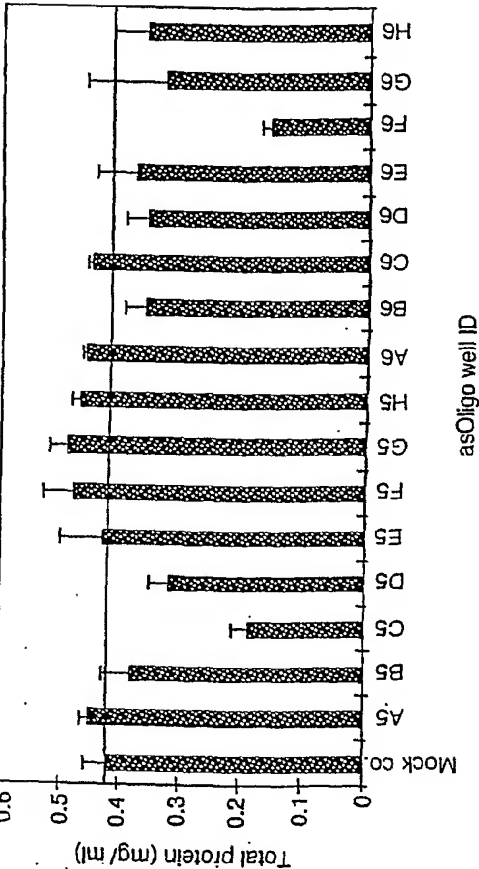




Figure 1G

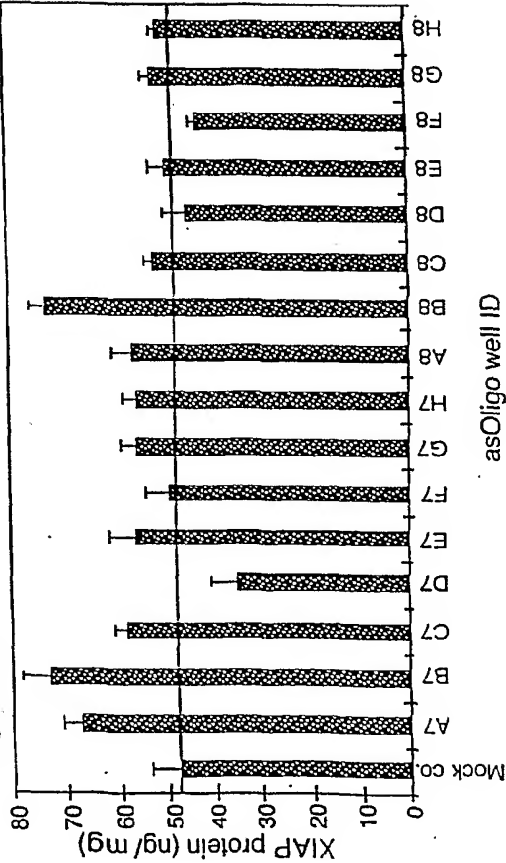


Figure 1H

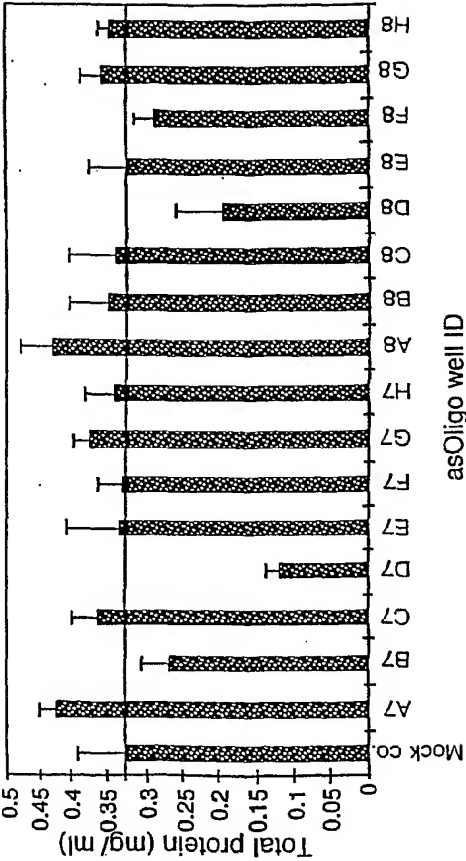


Figure 1I

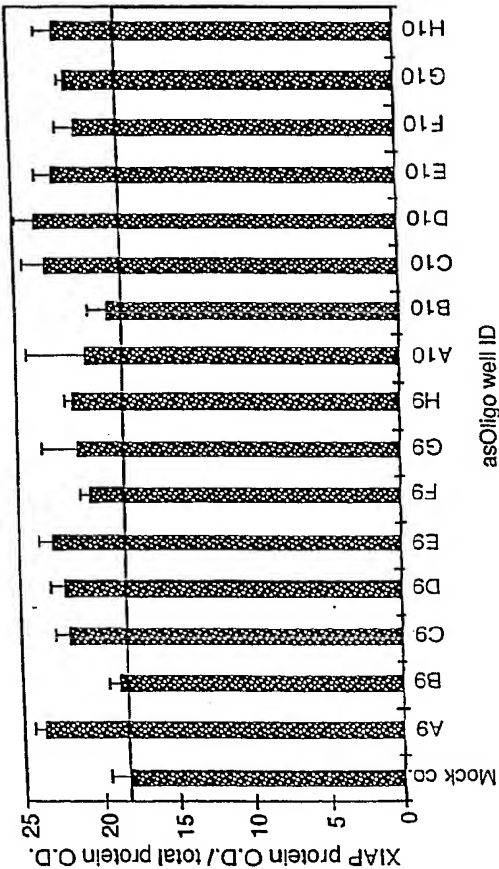


Figure 1J

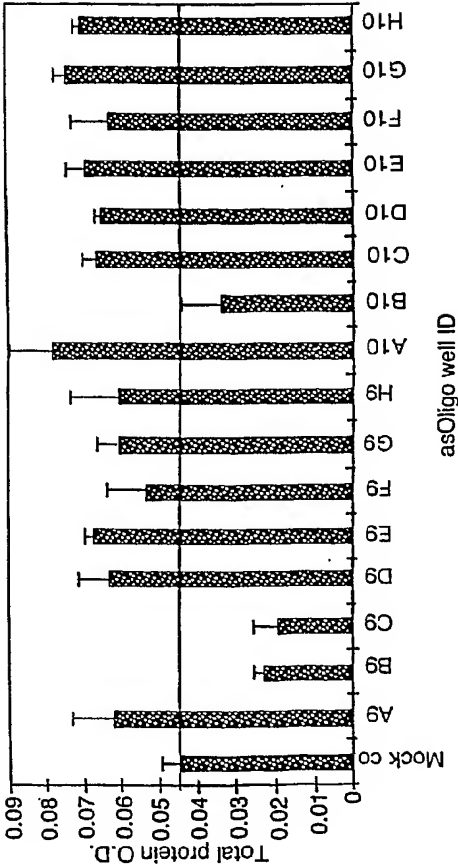


Figure 1K

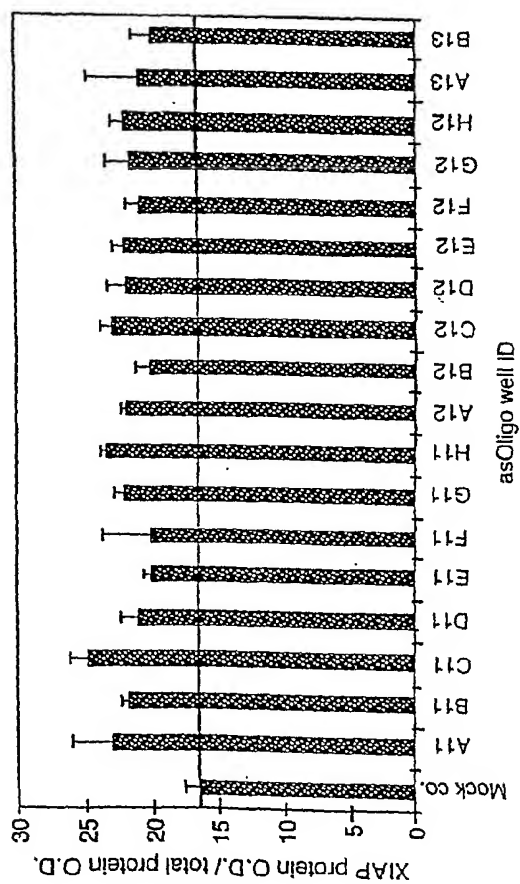


Figure 1L

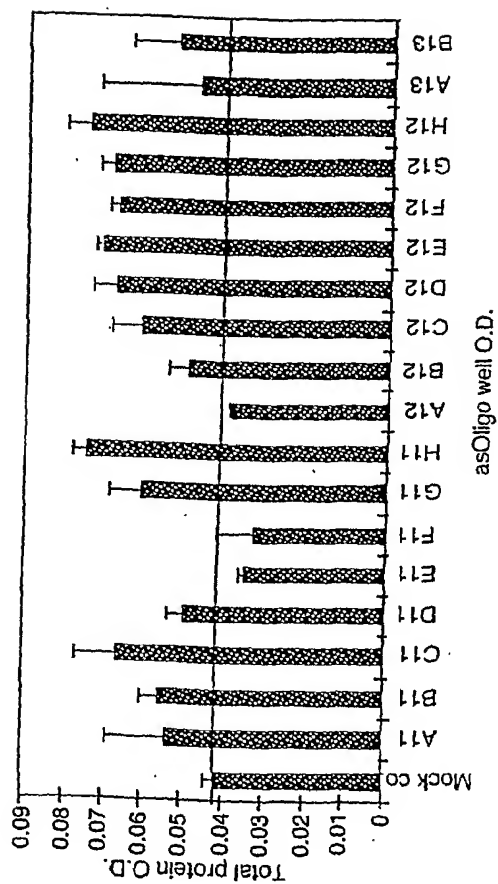


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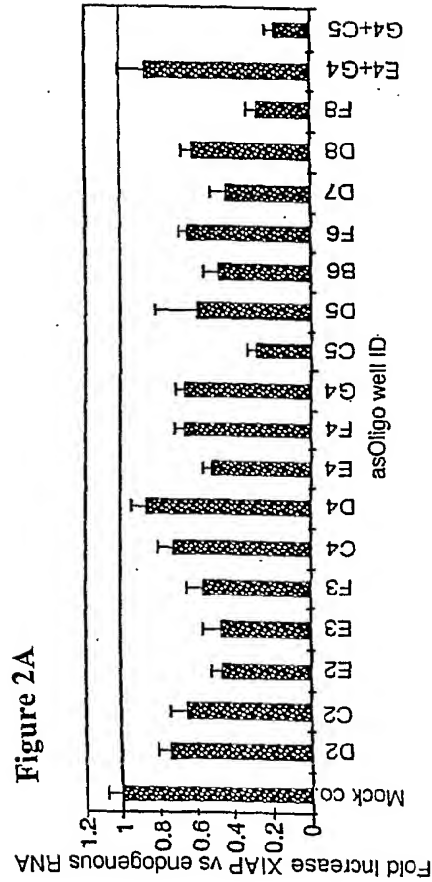


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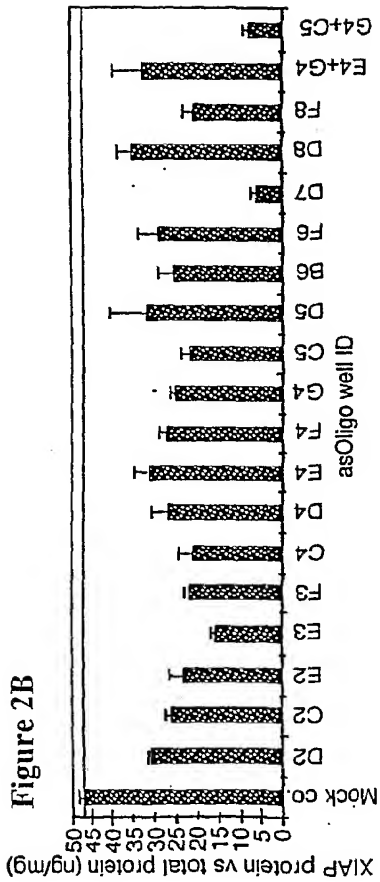


Figure 2C

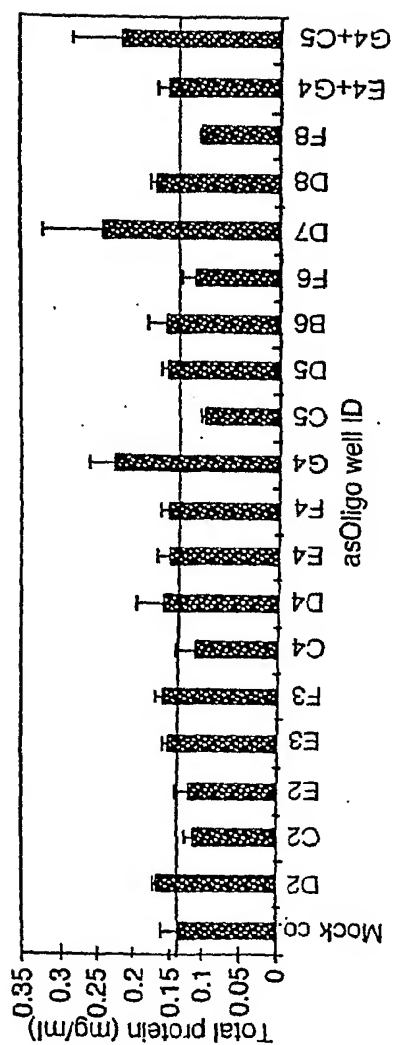


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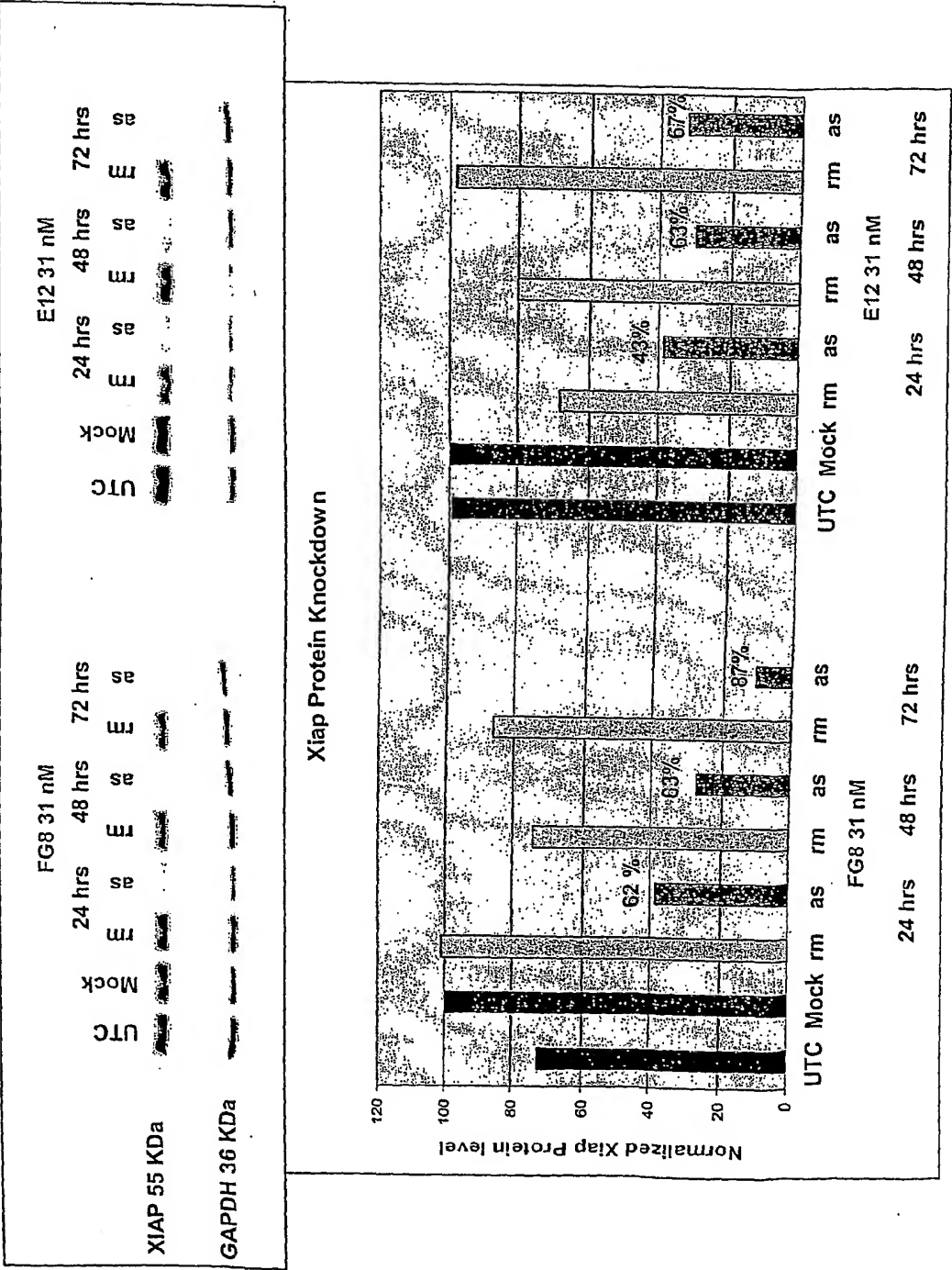


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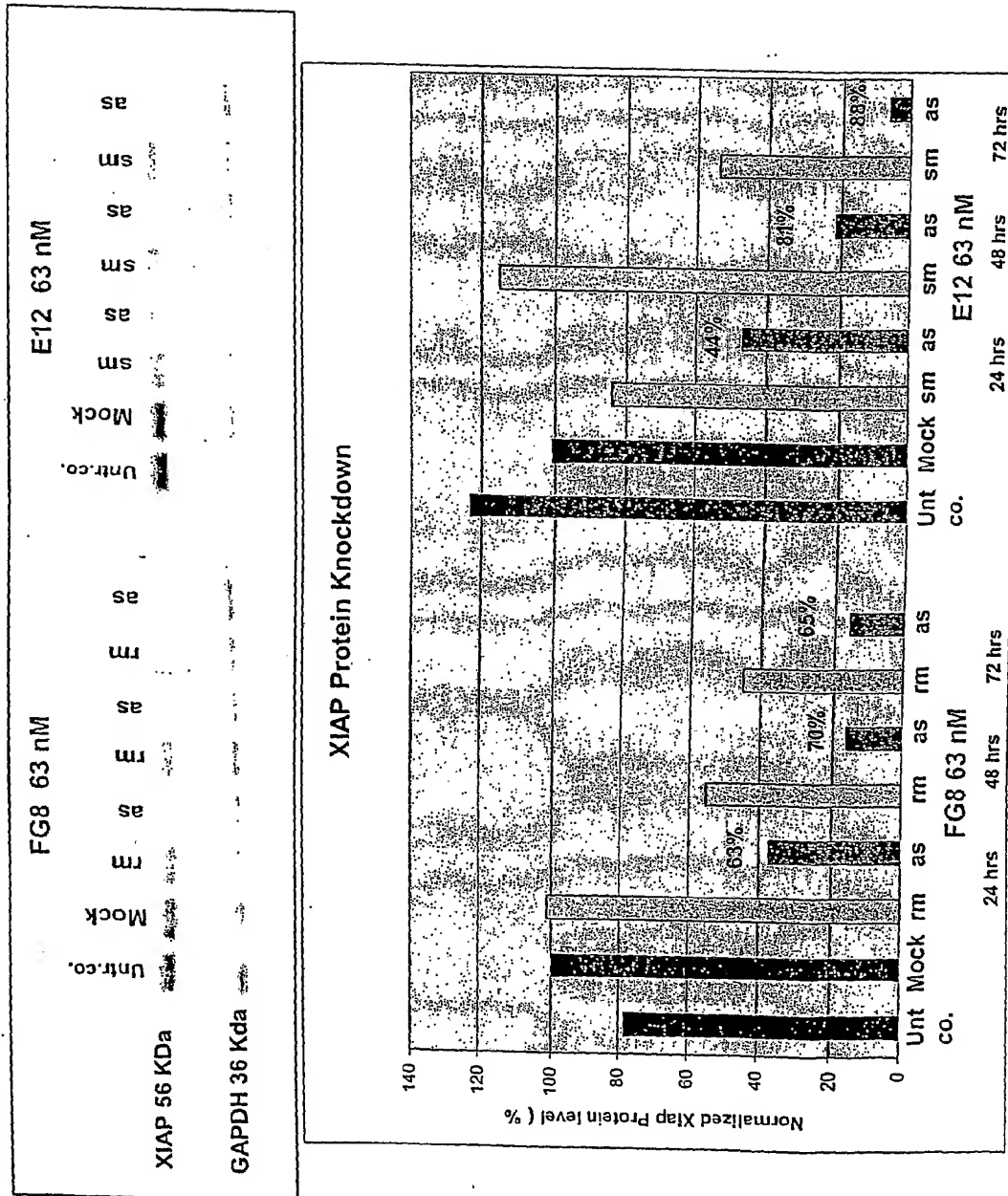


Figure 5A

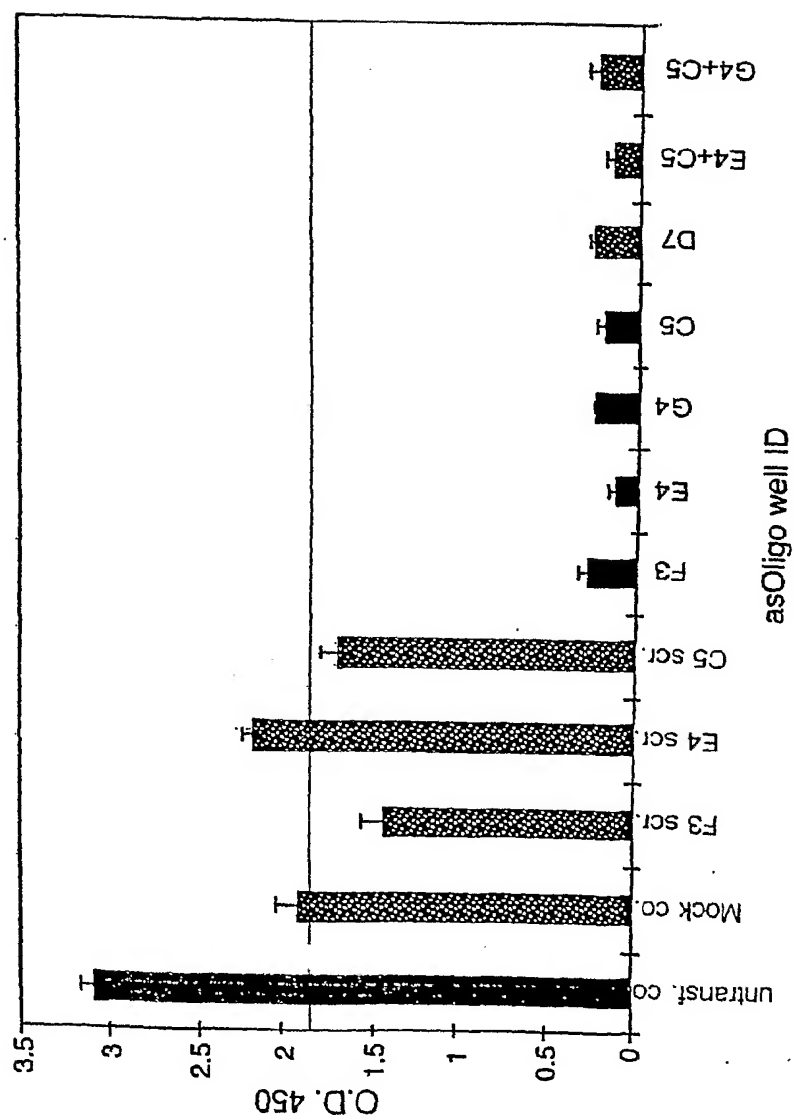




Figure 5B

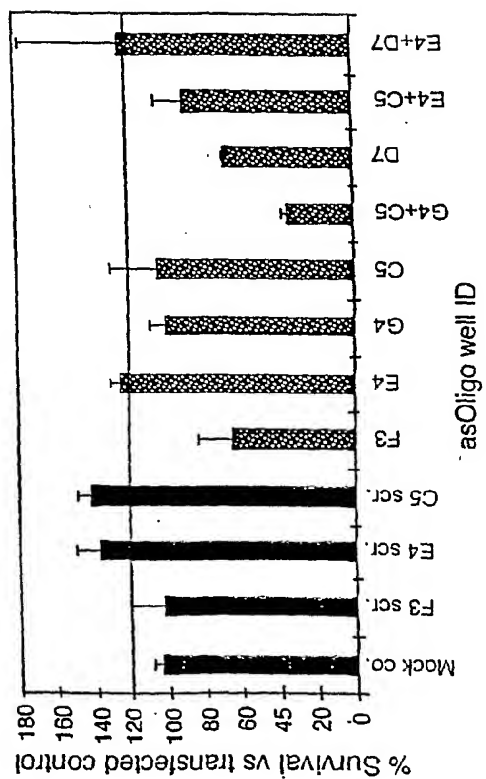


Figure 5C

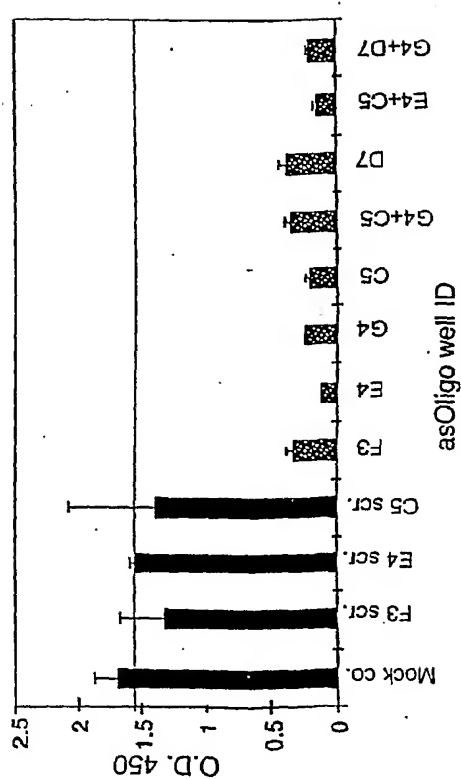
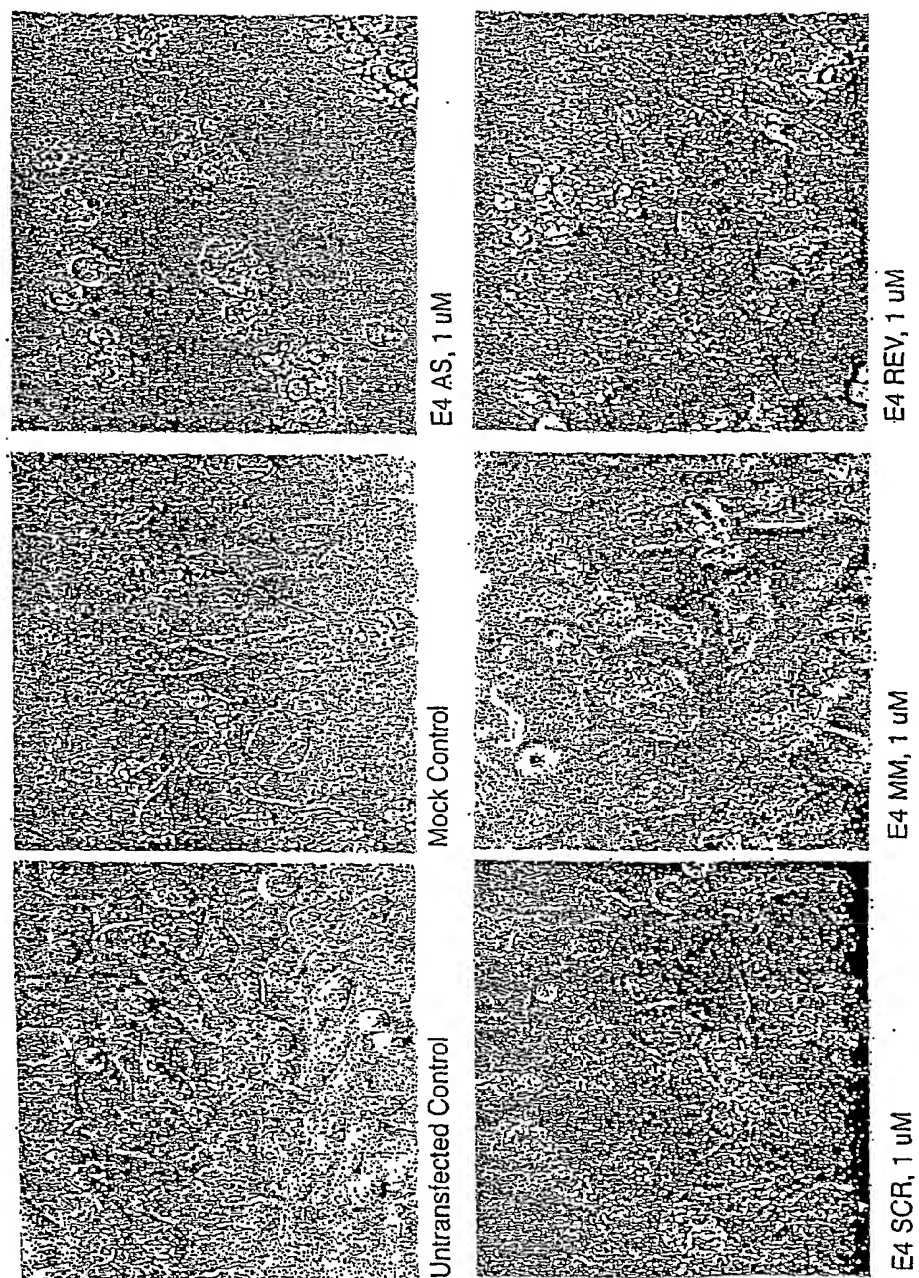


Figure 5D



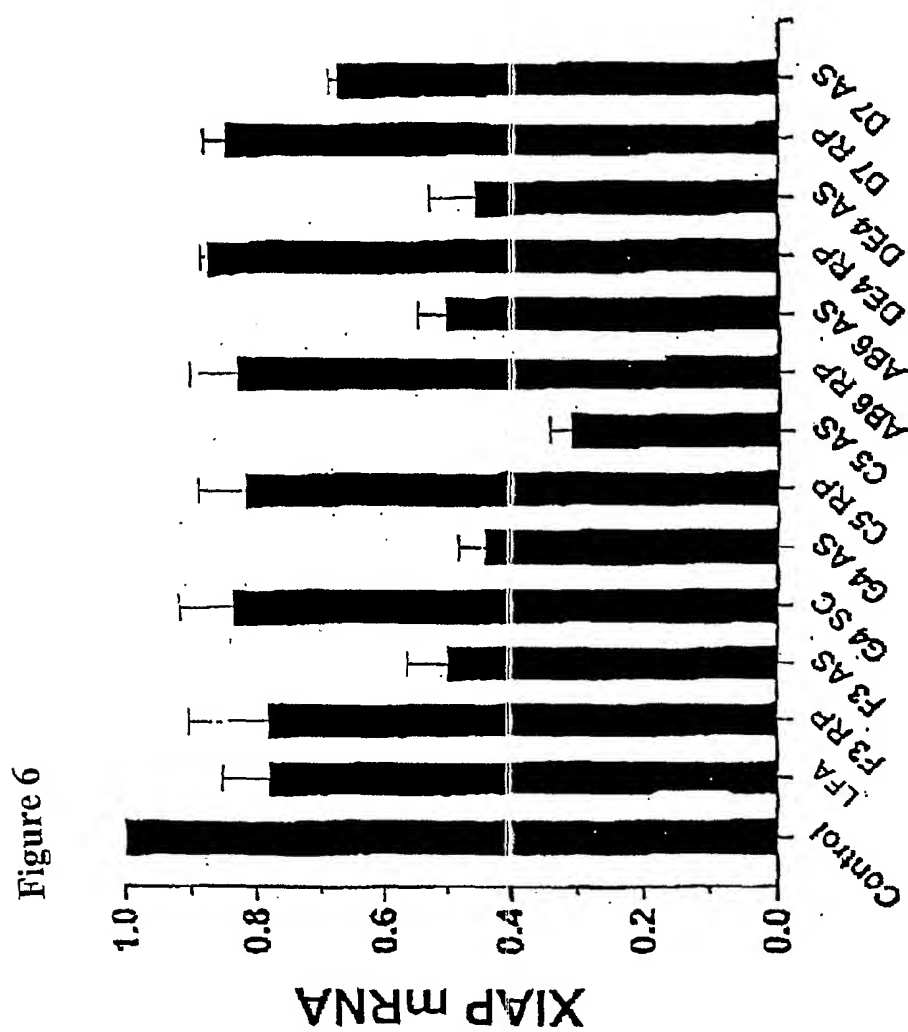


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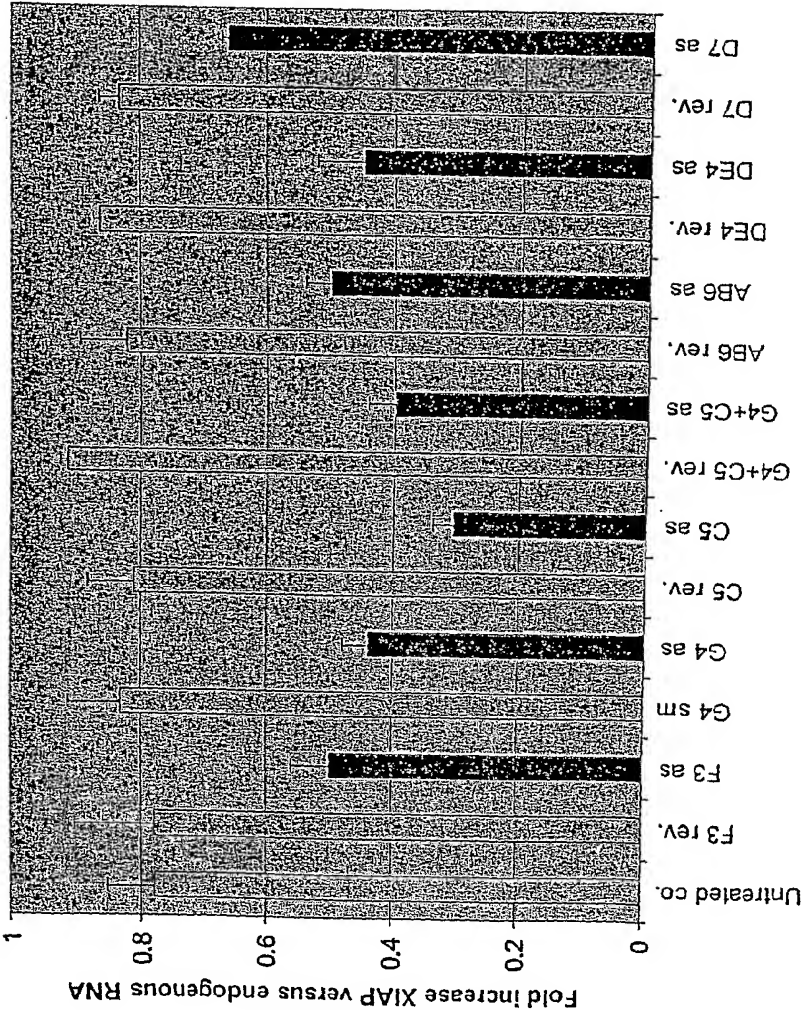


Figure 8

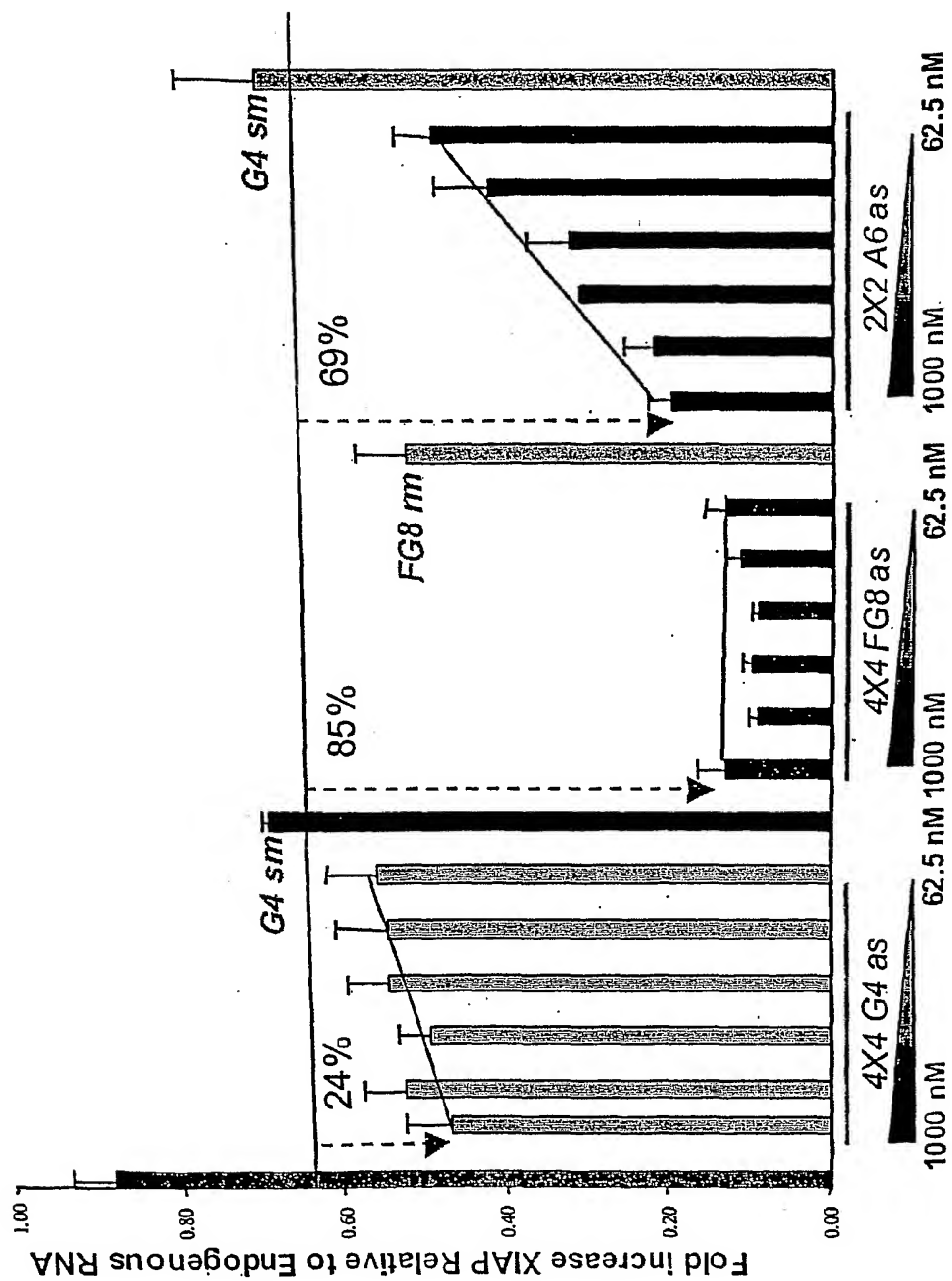


Figure 9

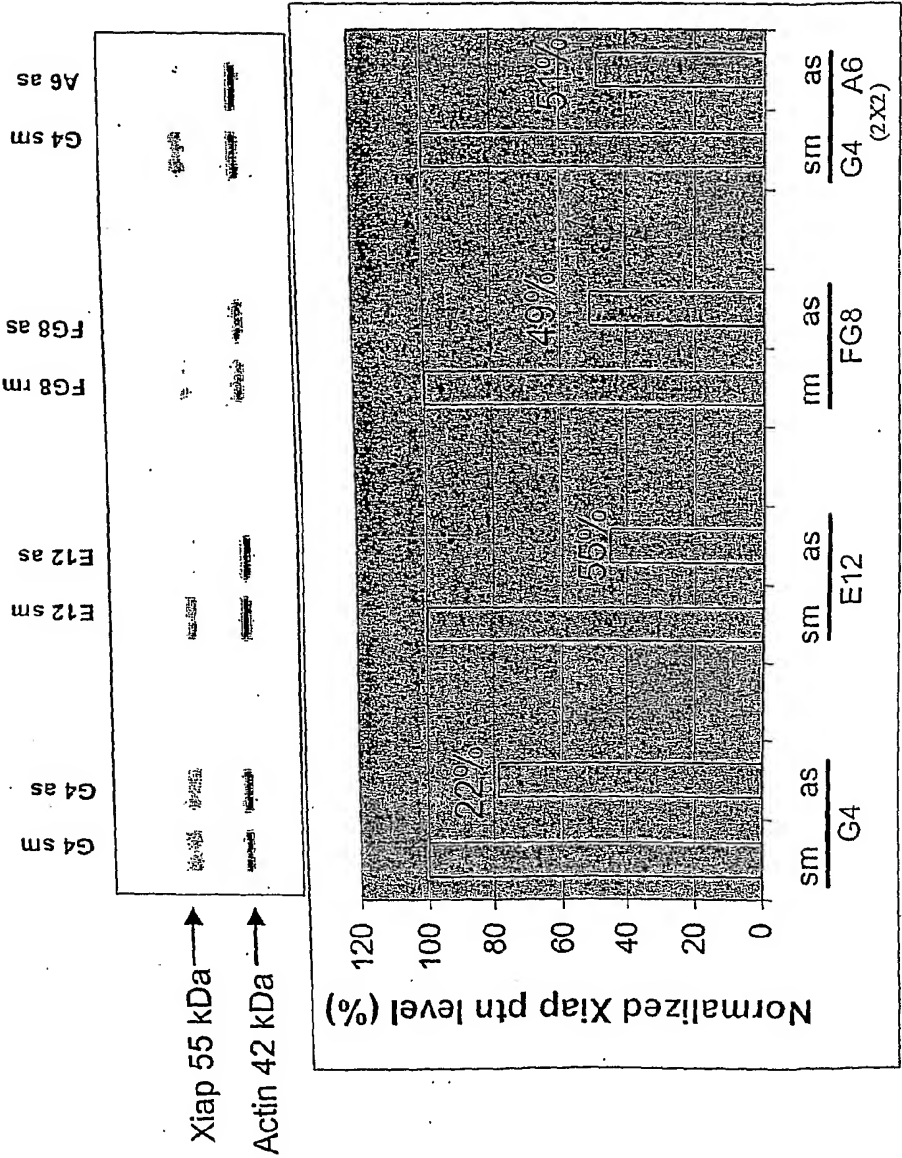


Figure 10A

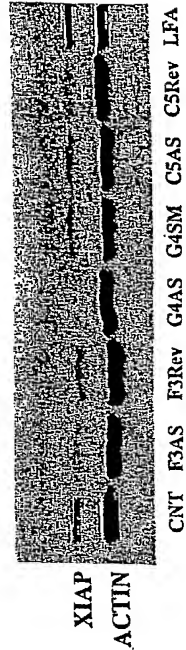


Figure 10B

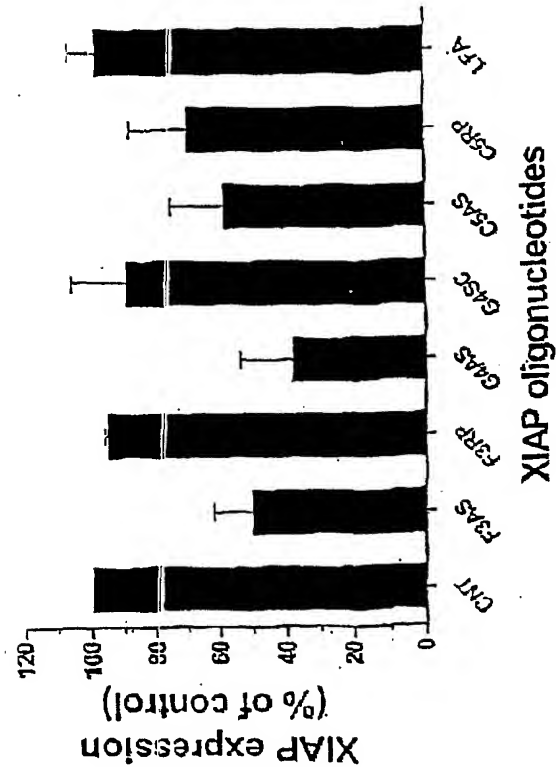


Figure 11A

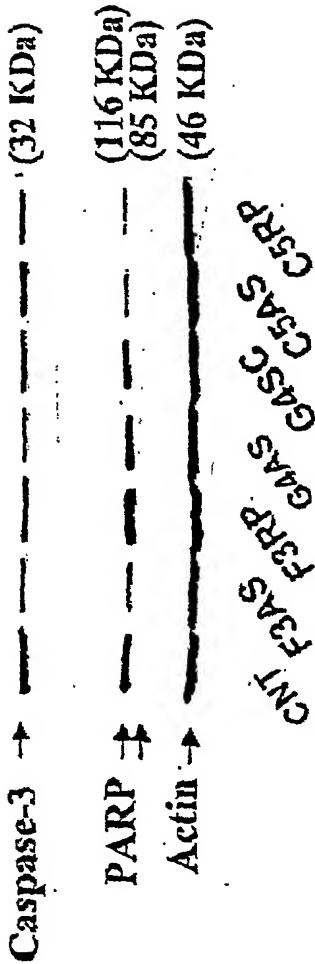


Figure 11B

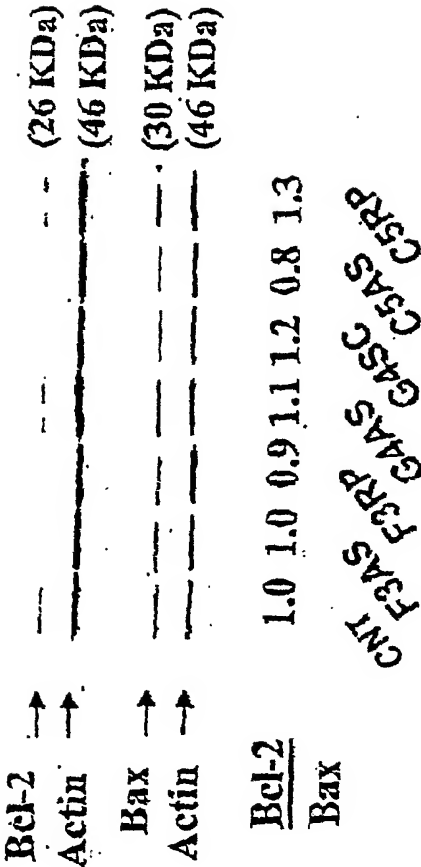




Figure 12

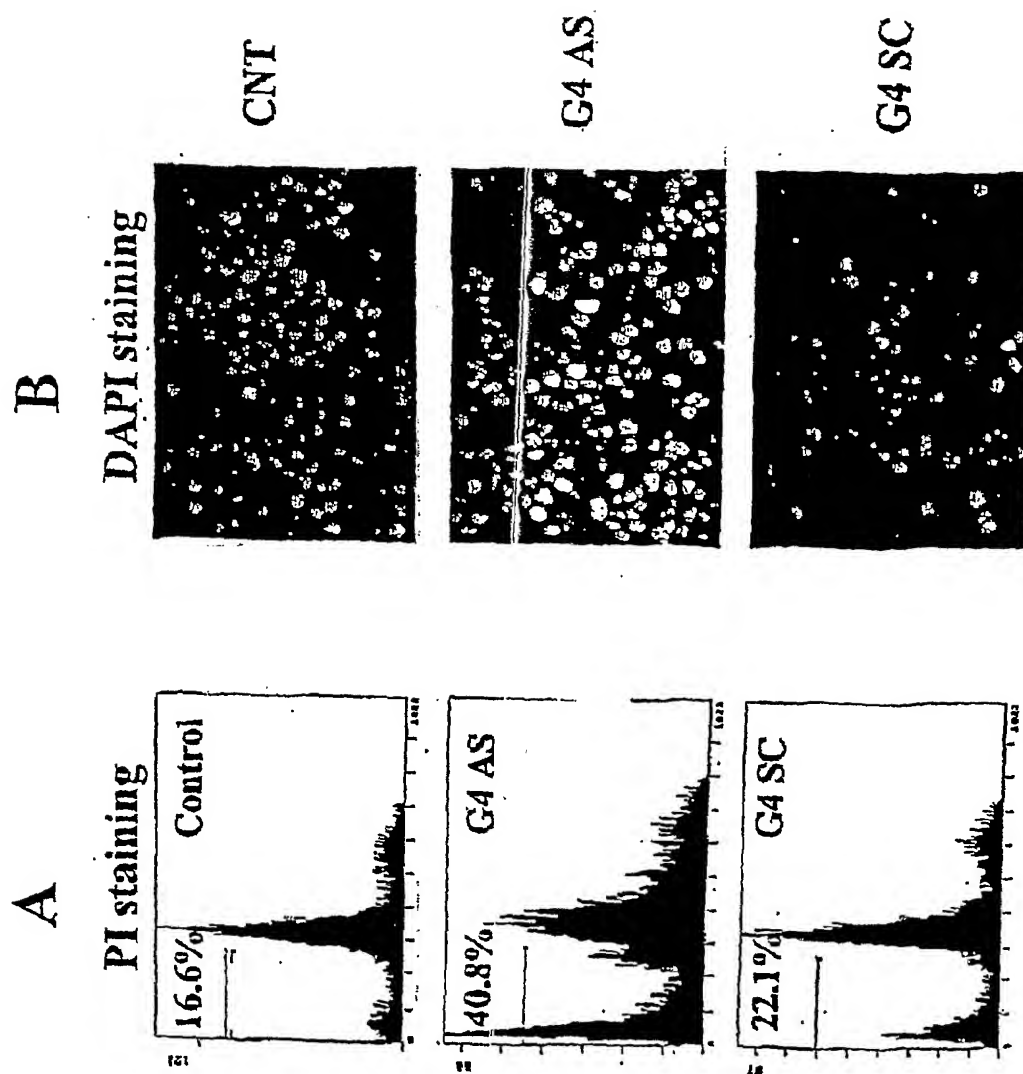


Figure 13A

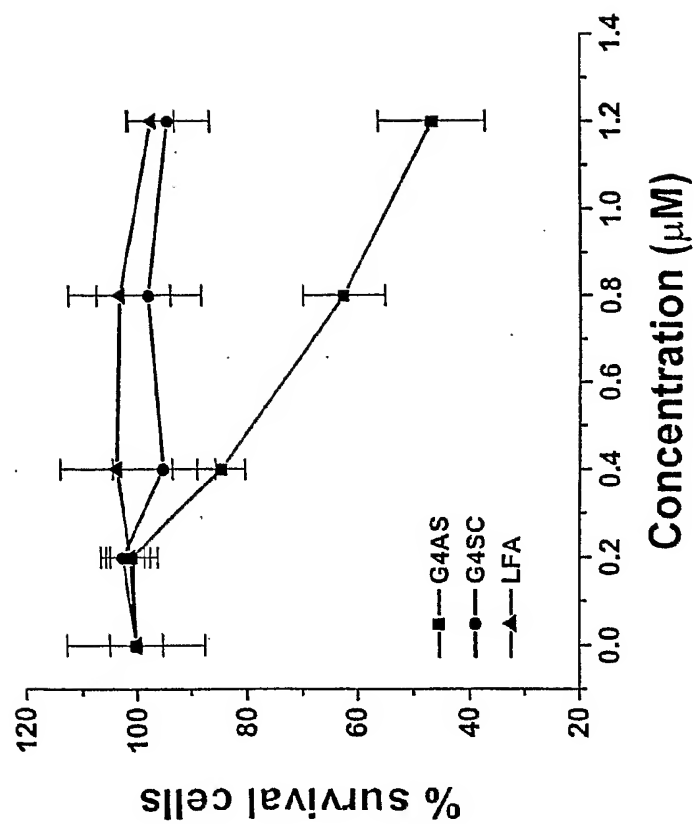


Figure 13B

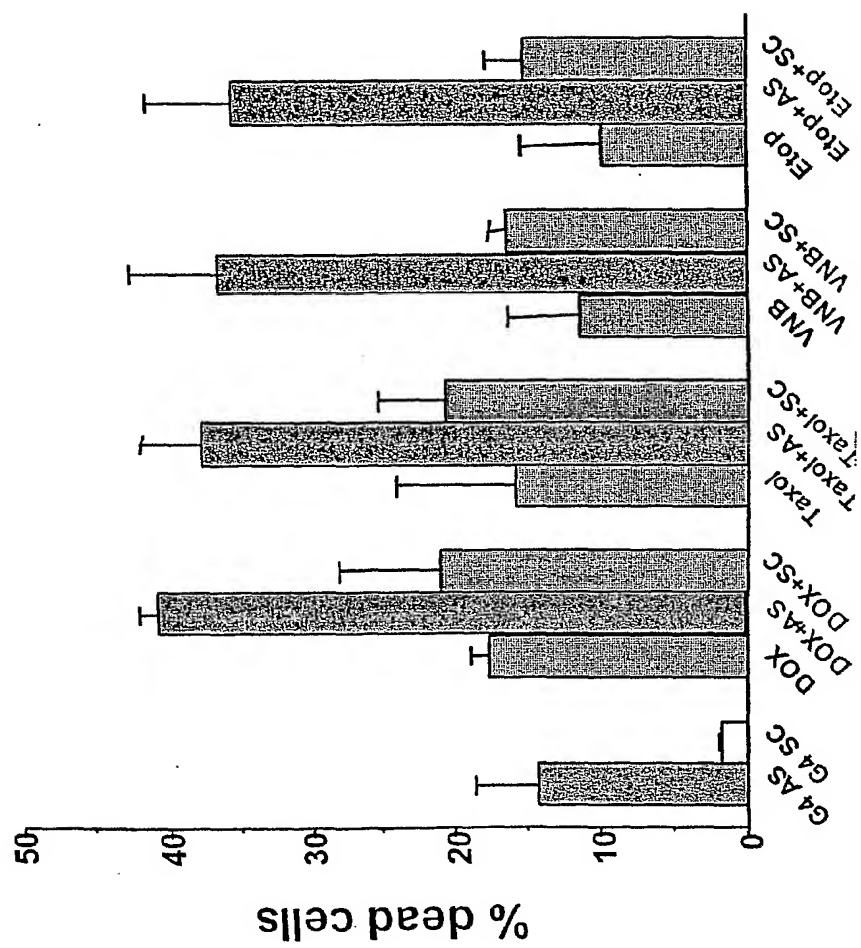


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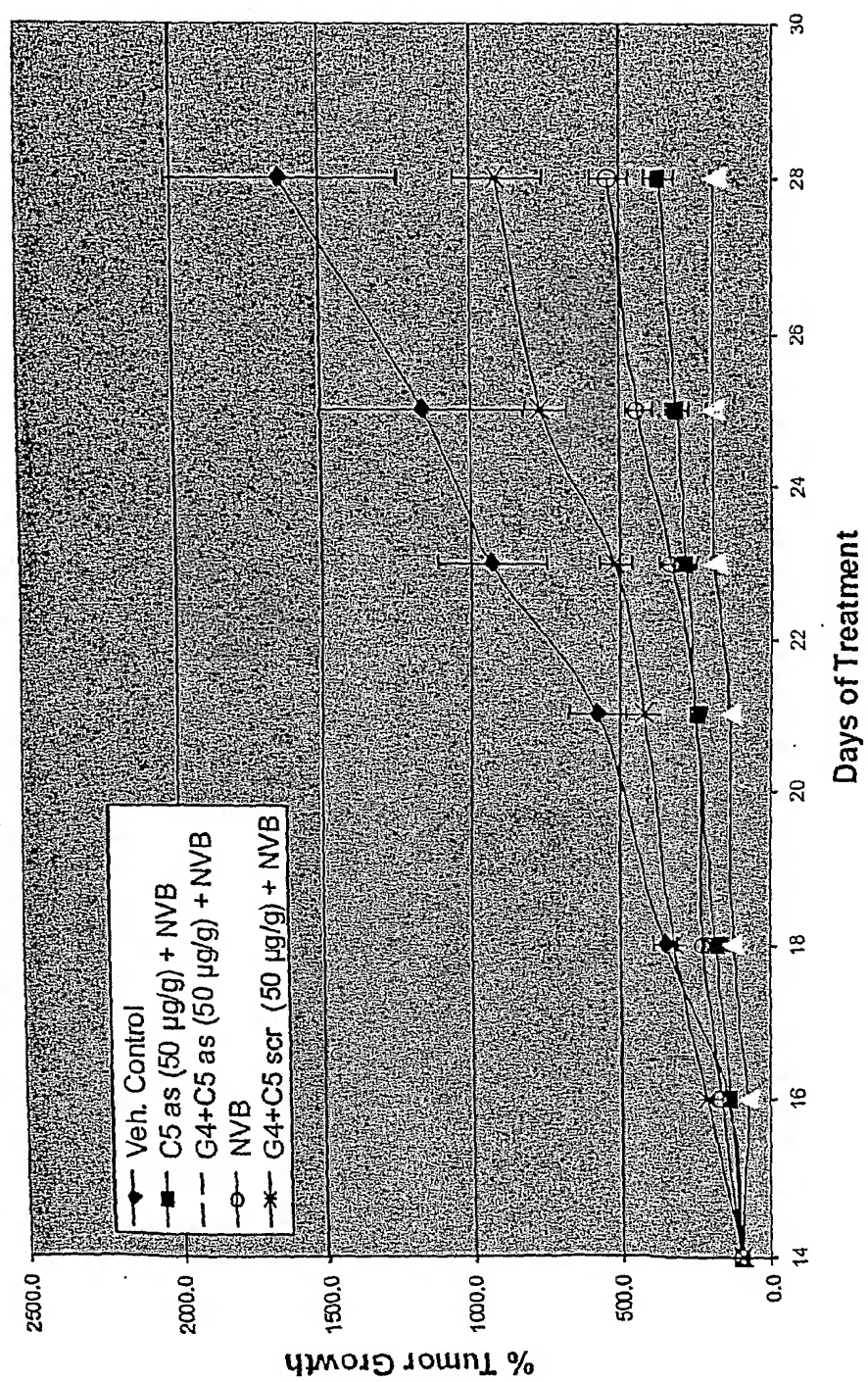


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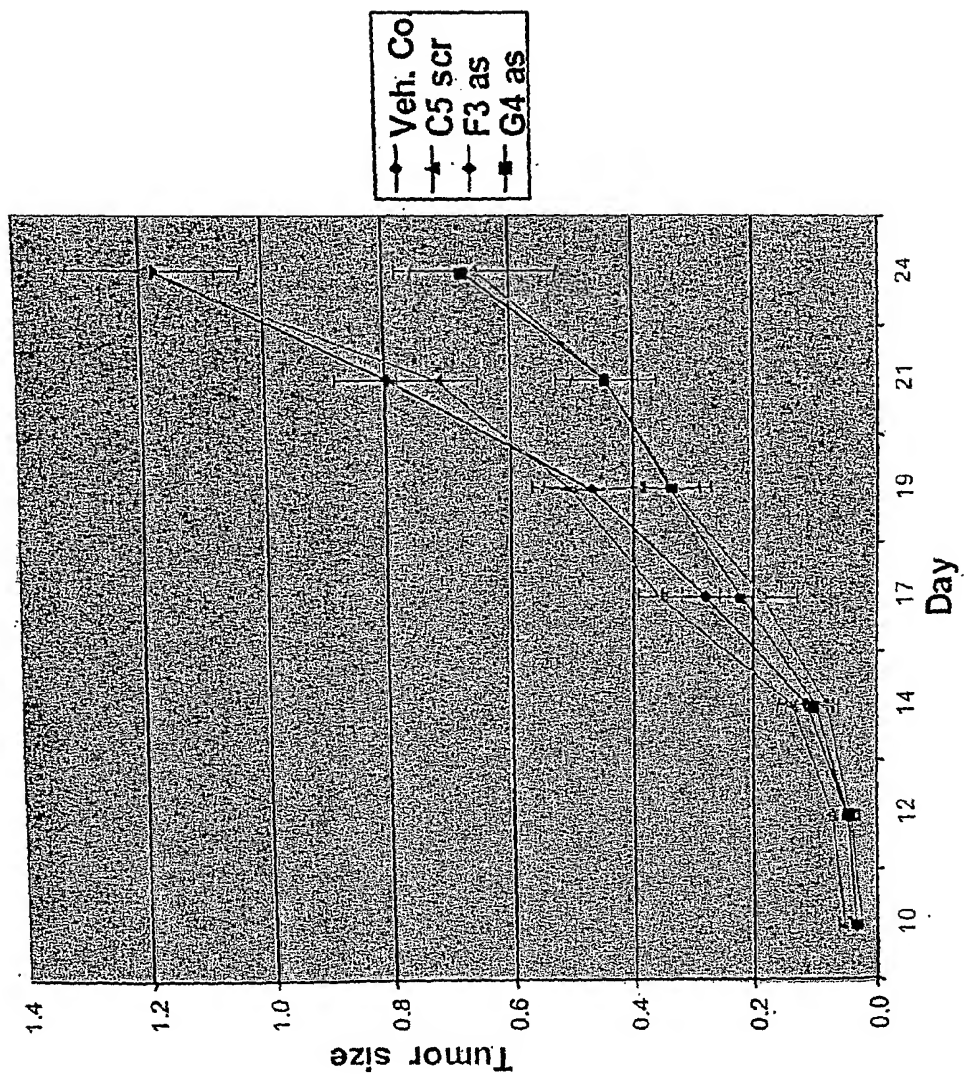


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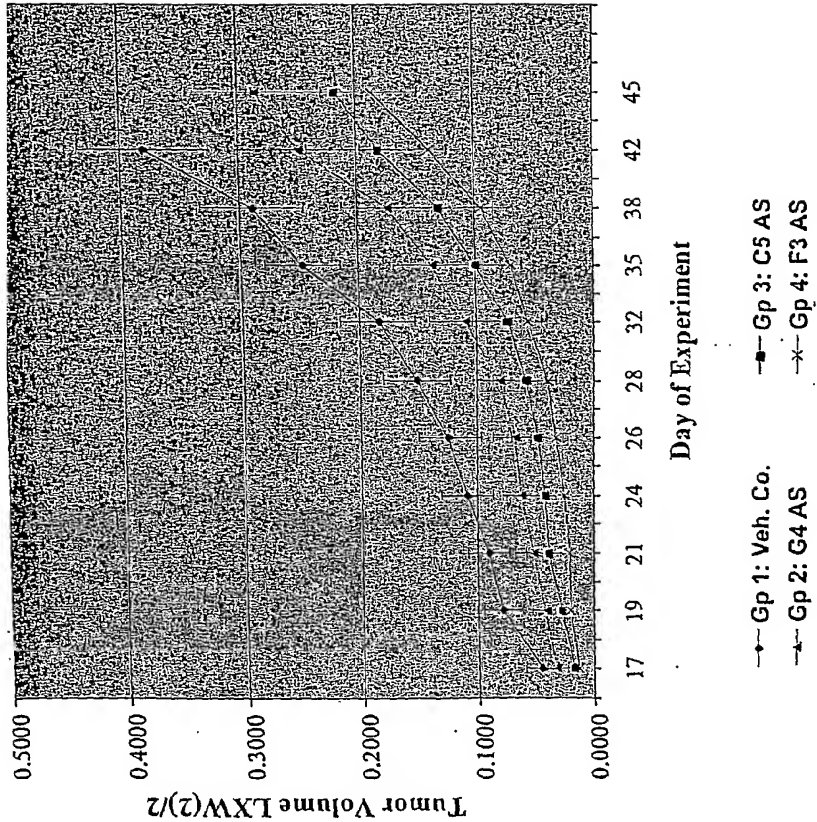


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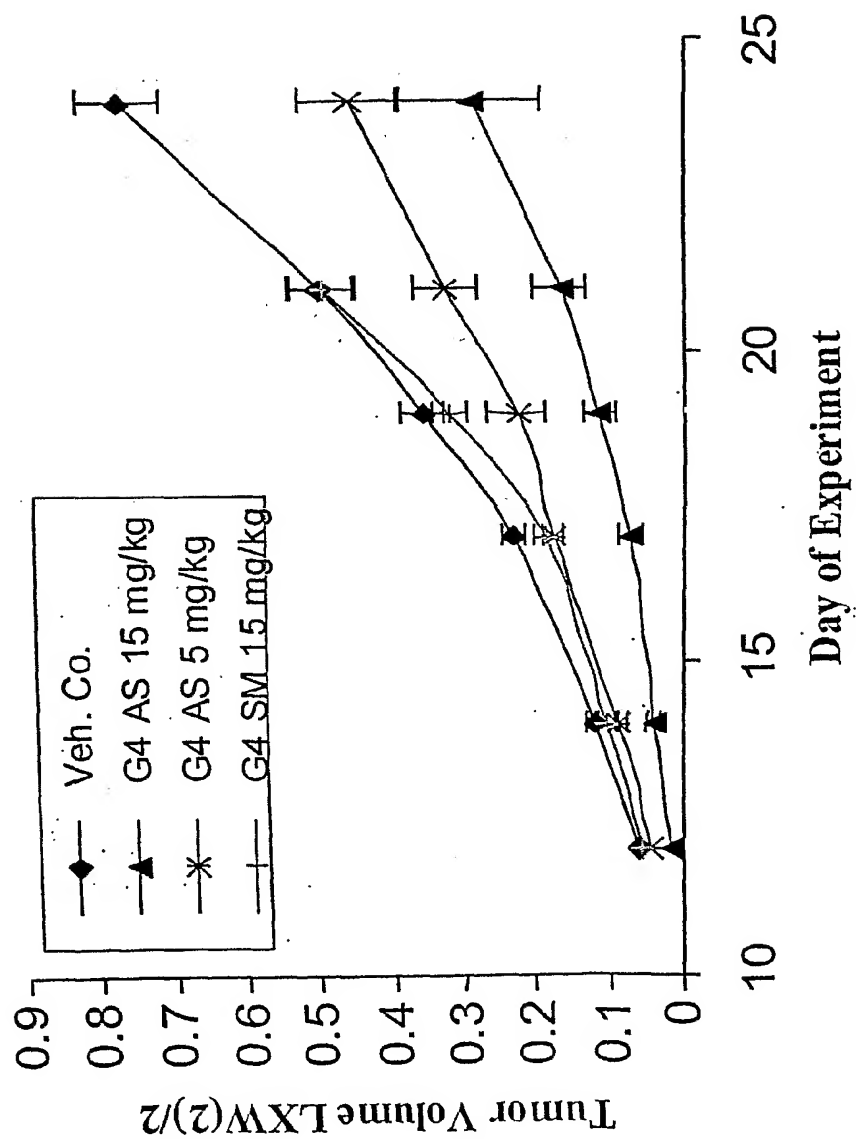


Figure 18A

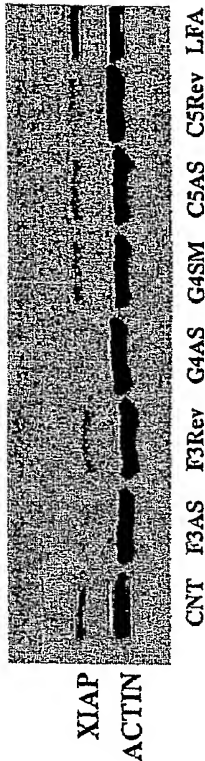


Figure 18B

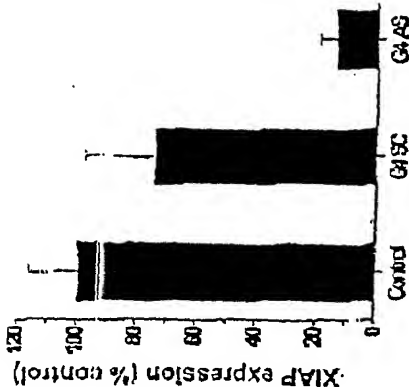




Figure 19A

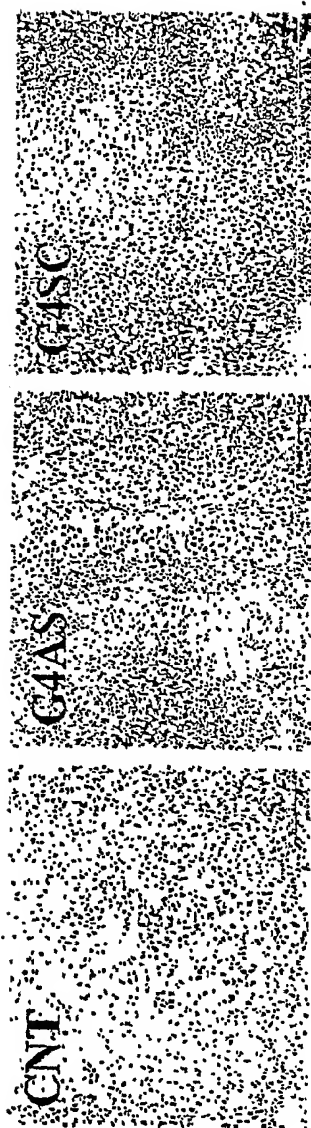


Figure 19B



Figure 20.

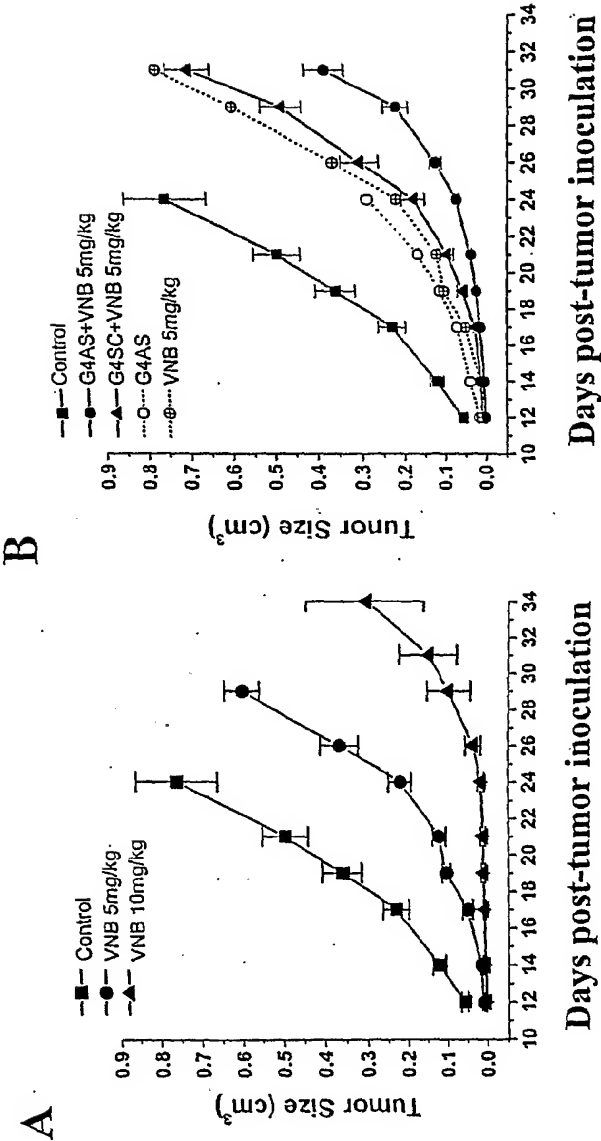


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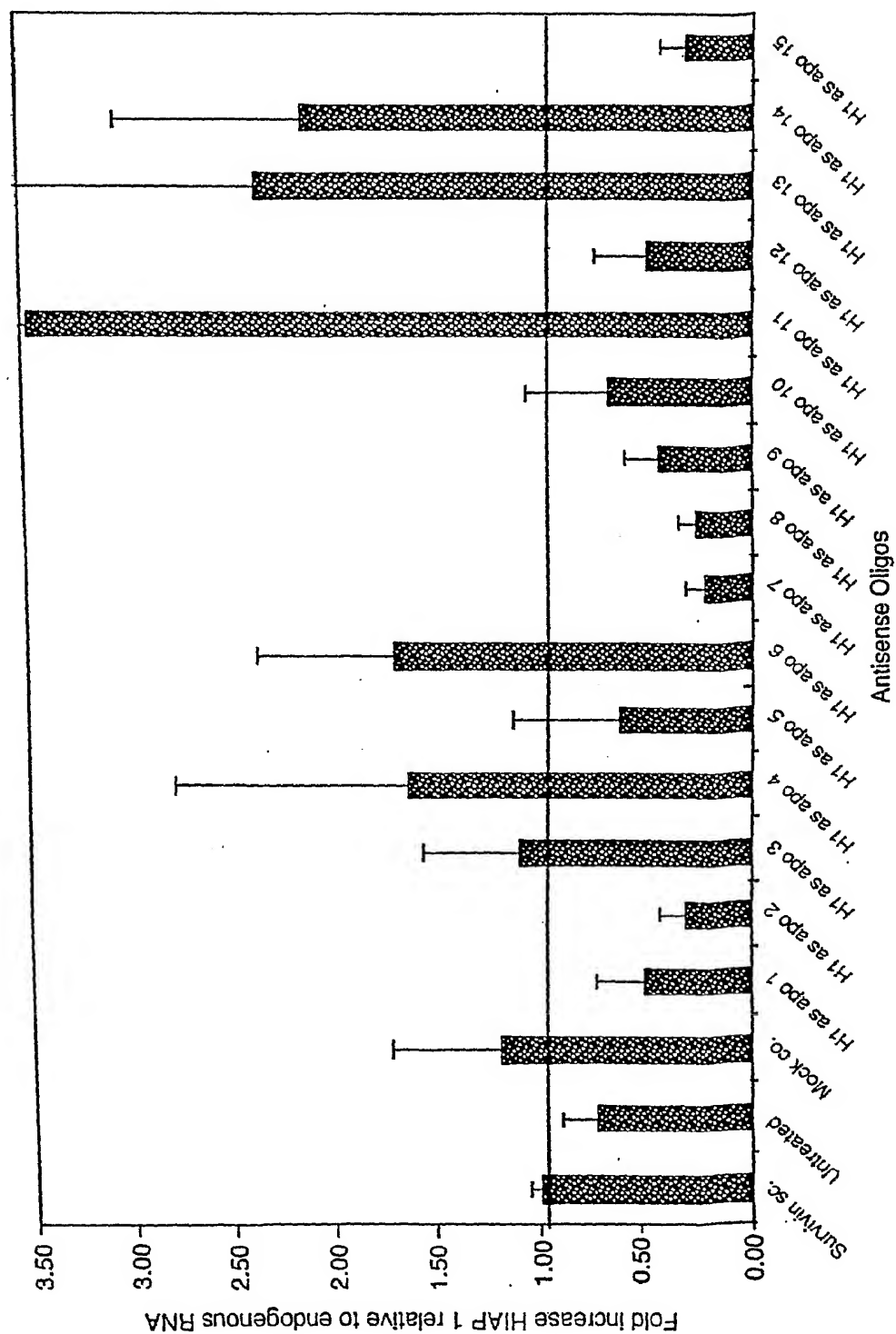


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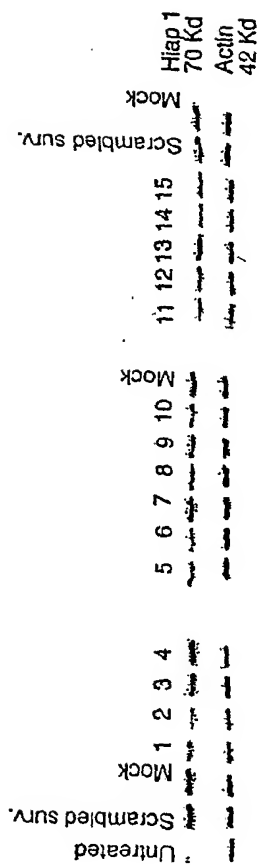


Figure 22B

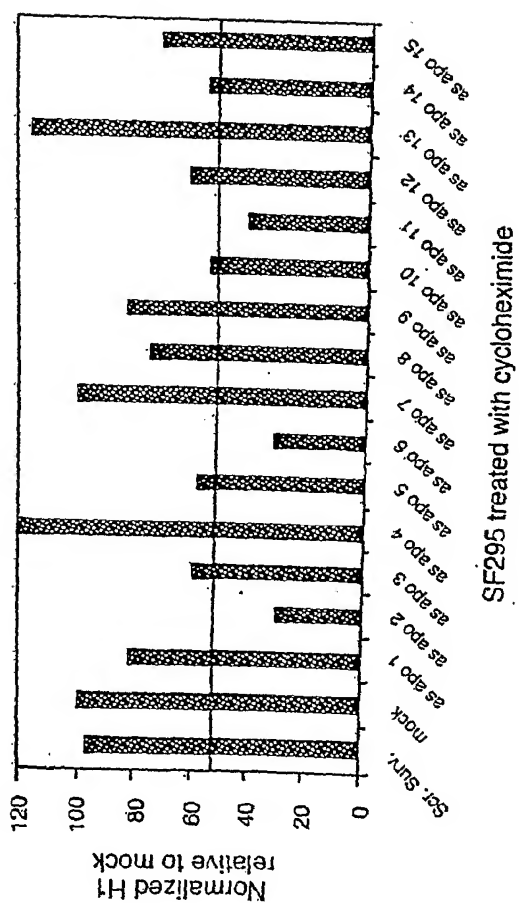


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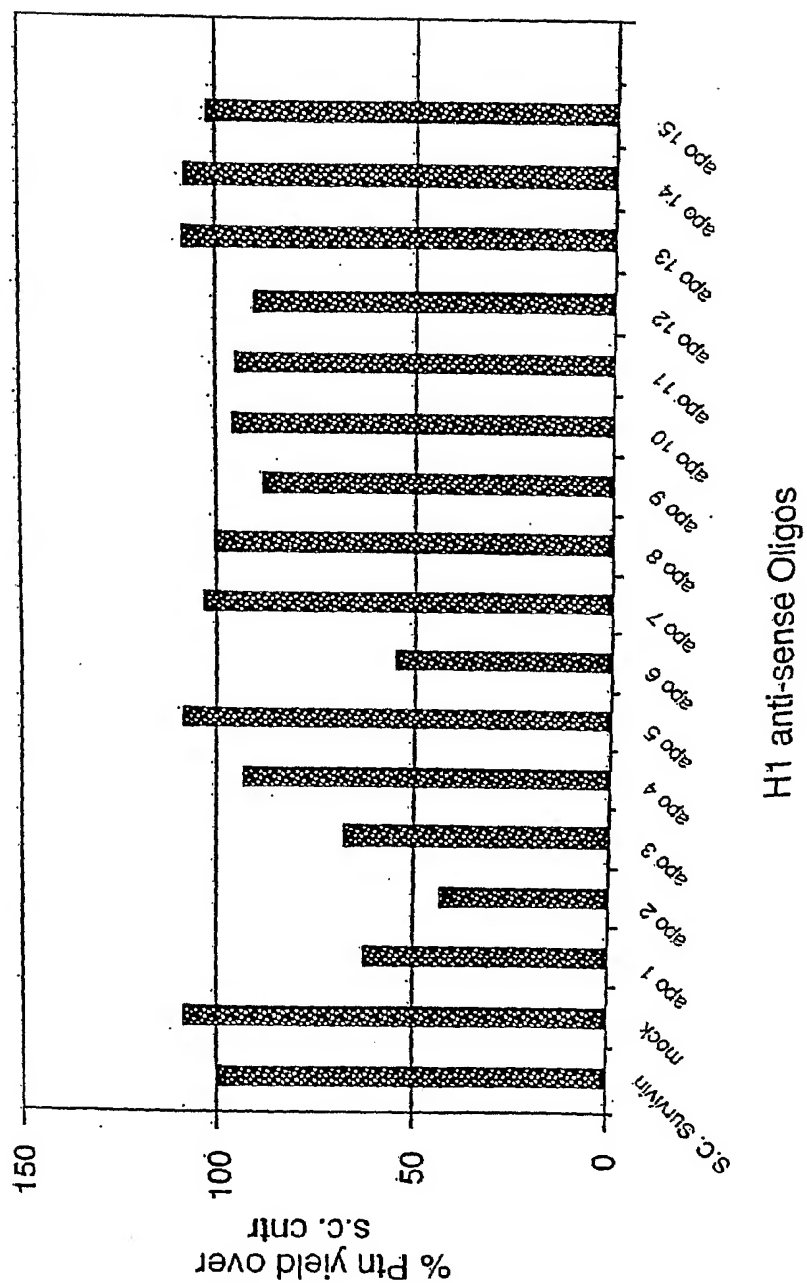


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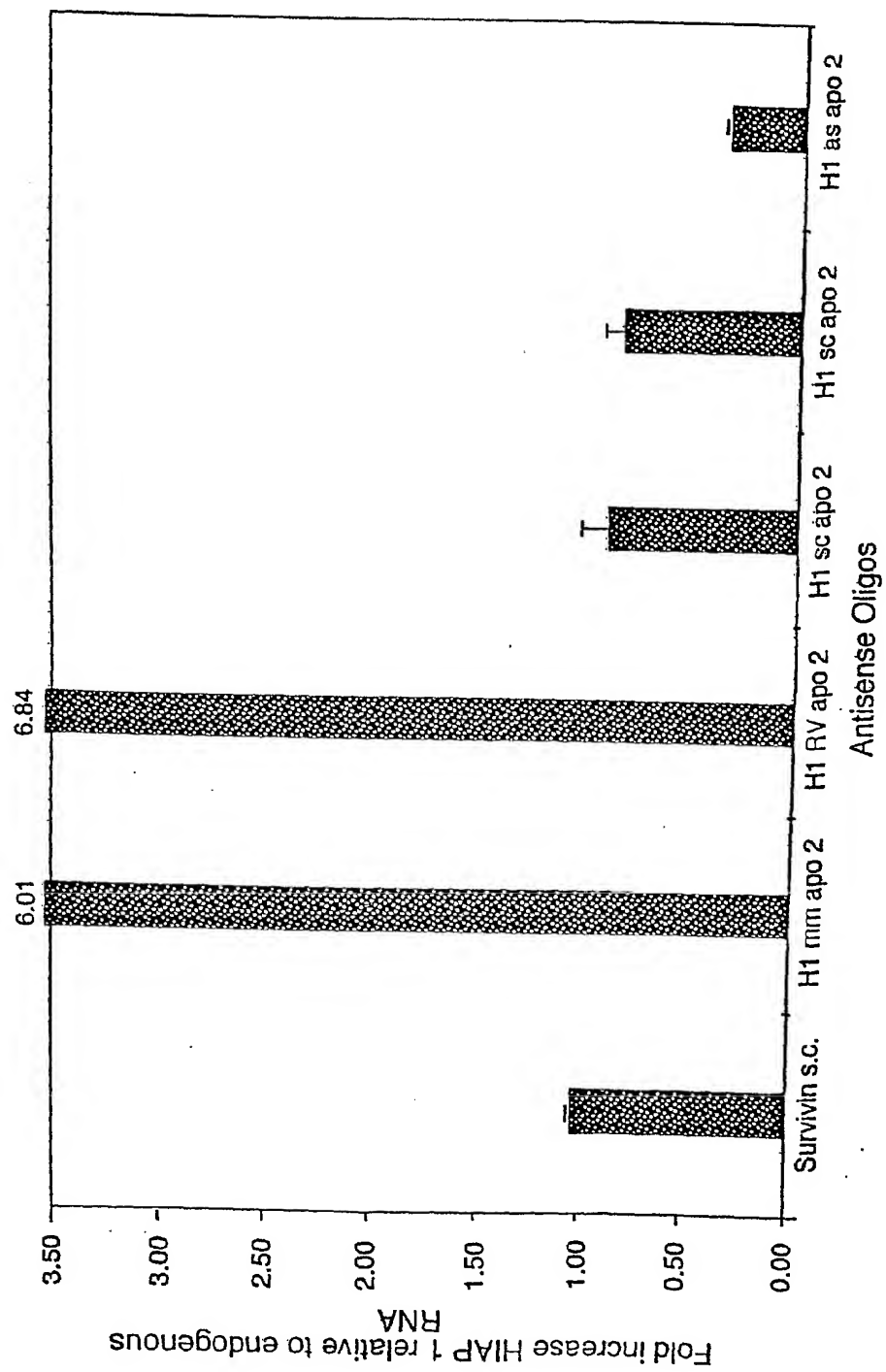
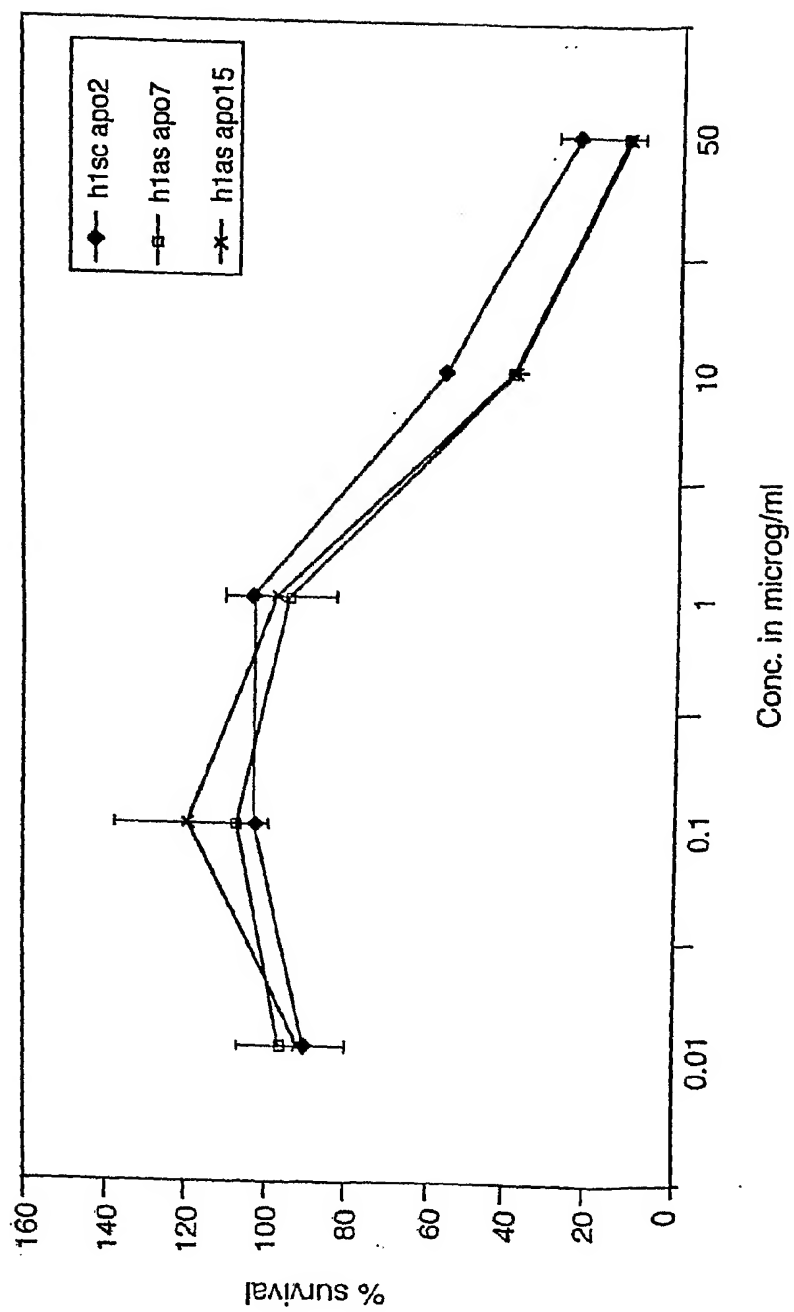


Figure 25



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Each nucleobase may be part of a ribonucleotide,



deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

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<223> n = T or U

<400> 5

agnangaaan annncngan

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<210> 6

<211> 19

<212> DNA

<213> Artificial Sequence

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 3, 6, 7, 12, 14, 16, 17, 19

<223> n = T or U

<400> 6

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<210> 7

<211> 19

<212> DNA

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 1, 2, 10, 12, 14, 15, 16, 17

<223> n = T or U

<400> 7

nnagcaaaa angnnnaa

19

<210> 8

<211> 19

<212> DNA

<213> Artificial Sequence

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 1, 5, 6, 9, 10, 11, 12, 13, 16, 18

<223> n = T or U

<400> 8

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<210> 9

<211> 19

<212> DNA

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 3, 11, 17, 18

<223> n = T or U

<400> 9

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19

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

<400> 10  
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deoxyribonucleotide, or nucleotide analog

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<223> n = T or U

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<210> 12  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

<400> 12  
gcagaanaaa acnaanaan

19

<210> 13  
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Each nucleobase may be part of a ribonucleotide,  
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<221> misc\_feature  
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<223> n = T or U

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<210> 14  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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<221> misc\_feature  
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<223> n = T or U

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<221> misc\_feature

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 <223> n = T or U

<400> 16  
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<210> 17  
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<221> misc\_feature  
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 <223> n = T or U

<400> 17  
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<210> 18  
 <211> 19  
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<221> misc\_feature  
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 <223> n = T or U

<400> 18  
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<210> 19  
 <211> 19  
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<220>  
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<221> misc\_feature  
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 <223> n = T or U

<400> 19  
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<210> 20  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 4, 5, 7, 9, 10, 17  
<223> n = T or U

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19

<210> 21  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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19

<210> 22  
<211> 19  
<212> DNA  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 4, 5, 7, 9, 10, 16  
<223> n = T or U

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19

<210> 23  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 11, 13, 14  
<223> n = T or U

<400> 23  
ccnncaaaaac ngnnaaaag

19

<210> 24  
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<212> DNA  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 4, 6, 12  
<223> n = T or U

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angncngcag gnacacaag

19

<210> 25  
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Each nucleobase may be part of a ribonucleotide,  
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<221> misc\_feature  
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19

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 8, 14, 15

<223> n = T or U

<400> 26

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19

<210> 27

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 1, 7, 9, 10, 13, 16

<223> n = T or U

<400> 27

ngccagngnn gangcngaa

19

<210> 28

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 4, 15, 18

<223> n = T or U

<400> 28

gnanaaagaa acccngcnc

19

<210> 29

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 8, 10, 12, 15, 16

<223> n = T or U

<400> 29

cgcacggnan cncnncac

19



<210> 30  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<400> 30  
cnacagcngc angacaacn

19

<210> 31  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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19

<210> 32  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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19

<210> 33  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 7, 9, 14, 15, 16  
<223> n = T or U

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19

<210> 34  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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19

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 5, 6, 8, 12  
<223> n = T or U

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accanncnng anaccagaa

19

<210> 36  
<211> 19  
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<220>  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 5, 6, 11, 12, 13, 15, 18  
<223> n = T or U

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agnnnncaac nnnnacng

19

<210> 37  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 5, 7, 9, 12, 13  
<223> n = T or U

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19

<210> 38  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 4, 9, 11, 13  
<223> n = T or U

<400> 38  
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19

<210> 39  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 6, 14, 17, 19  
<223> n = T or U

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19

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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19

<210> 41  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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19

<210> 42  
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deoxyribonucleotide, or nucleotide analog

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<223> n = T or U

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cagggnnccn cgggnanan

19

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 6, 7, 13, 17  
<223> n = T or U

<400> 43  
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19

<210> 44  
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<212> DNA  
<213> Artificial Sequence

<220>  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 7, 8, 10, 19  
<223> n = T or U

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ggccagnnncn gaaaggacn

19

<210> 45  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 7, 9, 11, 12, 17, 18  
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19

<210> 46  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 4, 7, 12  
<223> n = T or U

<400> 46  
gngnagnaga gnccagcac

19

<210> 47  
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<212> DNA  
<213> Artificial Sequence

<220>  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 7, 12, 13, 16  
<223> n = T or U

<400> 47  
aagcacngca cnnngncac

19

<210> 48  
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<213> Artificial Sequence

<220>  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 2, 6, 7, 8, 9  
<223> n = T or U

<400> 48  
nncagnnnnc caccacaac

19

<210> 49  
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<213> Artificial Sequence

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 5, 13, 14  
<223> n = T or U

<400> 49  
acgancacaa ggnncccaa

19

<210> 50  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 6, 8, 10, 11, 13  
<223> n = T or U

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ncgccngngn ncngaccag

19

<210> 51  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

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ccggcccaaa acaaagaag

19

<210> 52  
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<212> DNA  
<213> Artificial Sequence

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 8, 9, 14, 16, 17  
<223> n = T or U

<400> 52  
ganncacnnc gaanannaa

19

<210> 53  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 3, 10, 18  
<223> n = T or U

<400> 53  
nancagaacn cacagcanc

19

<210> 54  
<211> 19  
<212> DNA  
<213> Artificial Sequence

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 7, 8, 9, 11, 12, 16, 17, 18  
<223> n = T or U

<400> 54  
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<210> 55  
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<212> DNA  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 3, 8, 12, 16, 17, 18  
<223> n = T or U

<400> 55  
ncngccangg anggannnc

19

<210> 56  
<211> 19  
<212> DNA  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 4, 10, 14, 17, 18  
<223> n = T or U



<400> 56  
aagnaaagan ccgngcnnc

19

<210> 57  
<211> 19  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

<400> 57  
cngagnanan ccangnccc

19

<210> 58  
<211> 19  
<212> DNA  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 7, 10, 13, 14, 16, 17  
<223> n = T or U

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gcaagcngcn ccnngnnaa

19

<210> 59  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 7, 12, 18  
<223> n = T or U

<400> 59  
aaagcanaaa anccagcnc

19

<210> 60  
<211> 19  
<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 9, 10, 11, 14, 15, 16, 18

<223> n = T or U

<400> 60

gaaagcacnn nacnnnanc

19

<210> 61

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 3, 8, 9, 14, 18, 19

<223> n = T or U

<400> 61

acngggcnnc caancagnn

19

<210> 62

<211> 19

<212> DNA

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<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 3, 5, 6, 15, 17, 18

<223> n = T or U

<400> 62

gnngnnccca agggncnnc

19

<210> 63

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 5, 9, 14, 15, 16  
<223> n = T or U

<400> 63  
accnnggana ccannnagc

19

<210> 64  
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<212> DNA  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

<400> 64  
ngnncnaaca ganannngc

19

<210> 65  
<211> 19  
<212> DNA  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

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deoxyribonucleotide, or nucleotide analog

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<223> n = T or U

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<211> 19

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<223> n = T or U

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<223> n = T or U

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<210> 92

<211> 19

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<221> misc\_feature

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19

<210> 121

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<223> n = T or U

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19

<210> 122

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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
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<223> n = T or U

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<222> 6, 8, 14, 19  
<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<221> misc\_feature

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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
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<223> n = T or U

<400> 208

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19

<210> 209

<211> 19

<212> DNA

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<221> misc\_feature

<222> 1, 9, 11, 13, 18

<223> n = T or U

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ngggaaaang ncncaggng

19

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<213> Artificial Sequence

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<223> n = T or U

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<221> misc\_feature  
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<223> n = T or U

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<221> misc\_feature  
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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

<400> 216  
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<210> 217  
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<222> 3, 6, 10, 12, 13

<223> n = T or U

<400> 217

cancancacn gnnacccac

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<210> 218

<211> 19

<212> DNA

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Each nucleobase may be part of a ribonucleotide,  
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<221> misc\_feature

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<223> n = T or U

<400> 218

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19

<210> 219

<211> 19

<212> DNA

<213> Artificial Sequence

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Each nucleobase may be part of a ribonucleotide,  
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<221> misc\_feature

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<223> n = T or U

<400> 219

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19

<210> 220

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<223> n = T or U

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<223> n = T or U

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<210> 223  
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Each nucleobase may be part of a ribonucleotide,  
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<221> misc\_feature  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

<400> 225  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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<210> 227  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 3, 4, 5, 6, 9, 11, 14, 19

<223> n = T or U

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<210> 228

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<213> Artificial Sequence

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<223> n = T or U

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<221> misc\_feature

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<223> n = T or U

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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<221> misc\_feature  
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<223> n = T or U

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<221> misc\_feature  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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deoxyribonucleotide, or nucleotide analog

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

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<223> n = T or U

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19

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Each nucleobase may be part of a ribonucleotide,  
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<223> n = T or U

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nccncagng cncnnncnc

19

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

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<223> n = T or U

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<210> 240

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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<221> misc\_feature  
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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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<210> 243  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<221> misc\_feature  
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deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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<223> n = T or U

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cnaaaancgn ancaancag 19

<210> 247  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 4, 9, 11, 12, 13, 16, 17, 18, 19

<223> n = T or U

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ggcngcaana nnnccnnnn

19

<210> 248

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<213> Artificial Sequence

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<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

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<222> 6, 7, 8, 10, 14, 19

<223> n = T or U

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gagagnnncn gaanacagn

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<210> 249

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deoxyribonucleotide, or nucleotide analog

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<223> n = T or U

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<210> 250

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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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gaaacancnn cngngggaa

19

<210> 252  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
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Each nucleobase may be part of a ribonucleotide,  
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<221> misc\_feature  
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<223> n = T or U

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cnnncnnagnag ncnccgcaa

19

<210> 254  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 2, 4, 8, 15, 16, 17  
<223> n = T or U

<400> 254  
nngnccanac acacnnnac

19

<210> 255  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 8, 9, 14  
<223> n = T or U

<400> 255  
aaccaaanna gganaaaaag

19

<210> 256  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 4, 5, 8, 10, 13, 14, 15, 19  
<223> n = T or U

<400> 256  
angnnccanan ggnnnnagan

19

<210> 257  
<211> 19  
<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 1, 5, 6, 7, 8, 11, 12, 16, 17

<223> n = T or U

<400> 257

naagnnnnac nncacnnac

19

<210> 258

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 4, 5, 11, 13, 14, 17

<223> n = T or U

<400> 258

angnncccgg nannagnac

19

<210> 259

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 5, 10, 13, 14, 16, 18, 19

<223> n = T or U

<400> 259

gggcncaggn aanncncnn

19

<210> 260

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 9, 13, 14

<223> n = T or U

<400> 260

gcccaggang ganncaaac

19

<210> 261

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
DNA/RNA hybrid.

<400> 261

gagaagatga ctggtaaca

19

<210> 262

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
DNA/RNA hybrid

<221> misc\_feature

<222> 1, 17, 18

<223> n = T or U

<400> 262

ngtgctattc tgtgaann

18

<210> 263

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 263

tctgcttcaa ggagctggaa

20

<210> 264

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 264

gaaaggaaag cgcaaccg

18

<210> 265

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 265

agccagatga cgaccccata gaggaacata

30

<210> 266

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 266

tggagatgat ccatgggttc a

21

<210> 267

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 267

gaactcctgt cctttaattc ttatcaagt

29

<210> 268

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 268

ctcacacctt ggaaaccact tggcatg

27

<210> 269

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 269

ggtgataaag taaagtgctt tcactgt

27

<210> 270

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

<400> 270

tcagtagttc ttaccagaca ctccctcaa

28

<210> 271  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.

<400> 271  
caacatgcta aatggatatcc agggtgcaaa tatc 34

<210> 272  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.

<400> 272  
gaaggagaag gtcggagtc 19

<210> 273  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.

<400> 273  
gaagatggatg atgggattc 19

<210> 274  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.

<400> 274  
caagcttccc gttctcagcc 20

<210> 275  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens. Each nucleobase is part of  
a deoxyribonucleotide or ribonucleotide.

<400> 275  
cagagatttc atttaacgu 19

<210> 276  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.



Each nucleobase may be part of a  
deoxyribonucleotide or ribonucleotide

<400> 276  
cuacgctcgc catcgtuca 19

<210> 277  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase is part of a deoxyribonucleotide  
or ribonucleotide

<400> 277  
ugcccaagaa tactaguca 19

<210> 278  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 4, 5, 7, 9, 10, 16  
<223> n = T or U

<400> 278  
ancnnncnn gaaaanagg 19

<210> 279  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 4, 5, 7, 9, 10, 16  
<223> n = T or U

<400> 279  
ancnnncnn gaaaanagg 19

<210> 280  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 4, 10, 11, 13, 15, 16, 18  
<223> n = T or U

<400> 280  
gganaaaaagn ncncnnncna

19

<210> 281  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 7, 9, 13, 15, 16  
<223> n = T or U

<400> 281  
gcngagncnc cananngcc

19

<210> 282  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 7, 9, 13, 15, 16  
<223> n = T or U

<400> 282  
gcngagncnc cananngcc

19

<210> 283  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 4, 6, 7, 8, 15, 19  
<223> n = T or U

<400> 283  
ggcncnnngc ccacngaan

19

<210> 284  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 5, 6, 8, 12  
<223> n = T or U

<400> 284  
accanncnngg anaccagaa

19

<210> 285  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 5, 6, 8, 12  
<223> n = T or U

<400> 285  
accanncnngg anaccagaa

19

<210> 286  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 8, 12, 14, 15  
<223> n = T or U

<400> 286  
aagaccanag gncnnacca

19

<210> 287  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 4, 5, 8, 13, 15, 17  
<223> n = T or U

<400> 287  
gggnnccncg ggnanangg

19

<210> 288  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 5, 7, 12, 15, 16  
<223> n = T or U

<400> 288  
ggnananggc gnccnnggg

19

<210> 289  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 5, 7, 10, 11, 18  
<223> n = T or U

<400> 289  
ggnancnccn ncaccagna

19

<210> 290  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 9, 10, 13, 15, 17  
<223> n = T or U

<400> 290  
angaccacnn ccncnangg

19

<210> 291  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 8, 9, 14, 16, 17  
<223> n = T or U

<400> 291  
ganncacnnc gaanannaa

19

<210> 292  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 6, 11, 12, 16, 17  
<223> n = T or U

<400> 292  
aannanaacg nncacnnag

19

<210> 293  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.

<400> 293  
ggtgataaag taaagtgcctt tcactgt

27

<210> 294

<211> 33  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> based on Homo sapiens.

<400> 294  
 caacatgcta aatgggttcca ggggtgcaa atc

33

<210> 295  
 <211> 28  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> based on Homo sapiens.

<400> 295  
 tcagtagttc ttaccagaca ctctctcaa

28

<210> 296  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> based on Homo sapiens.  
 DNA/RNA hybrid.

<400> 296  
 uaagctgttc tatgtguuc

19

<210> 297  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> based on Homo sapiens.

<400> 297  
 aagggcggcg gaggtagac

19

<210> 298  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> based on Homo sapiens.

<400> 298  
 agaggacgga gtcggaggc

19

<210> 299  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> based on Homo sapiens.

=

<400> 299  
cggagcgtga ggatggaga 19

<210> 300  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 18  
<223> n = T or U

<400> 300  
agcaaggaca agcccagnc 19

<210> 301  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 3, 9, 12  
<223> n = T or U

<400> 301  
ngnaaacccng cngcccaga 19

<210> 302  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 6, 9, 10, 11, 12, 15, 18, 19  
<223> n = T or U

<400> 302  
agaagncgnn ncccncnn 19

<210> 303  
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<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 7, 8, 13, 17

<223> n = T or U

<400> 303

ccgagannag acnaagncc

19

<210> 304

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 3, 4, 5, 6, 9, 10, 11, 13, 14, 15

<223> n = T or U

<400> 304

acnnnnccnn nannnccac

19

<210> 305

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 1, 14, 17, 19

<223> n = T or U

<400> 305

ncccaaacac aggnacnan

19

<210> 306

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog



<221> misc\_feature

<222> 3, 4, 6, 13

<223> n = T or U

<400> 306

cannncagc ggnaacagc

19

<210> 307

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 5, 8, 9, 11, 14, 17

<223> n = T or U

<400> 307

accancannc ncancncna

19

<210> 308

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 3, 5, 10, 11, 18

<223> n = T or U

<400> 308

aangnaaccn ncaaccanc

19

<210> 309

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.

Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 1, 2, 3, 5, 7, 8, 11, 15, 17

<223> n = T or U

<400> 309  
nnngnannca ncacngnc

18

<210> 310  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 6, 8, 11, 12  
<223> n = T or U

<400> 310  
ncacancnca nnaccaac

18

<210> 311  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 6  
<223> n = T or U

<400> 311  
ccaggnggca ggagaaaca

19

<210> 312  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 8, 9, 13, 16, 17, 18  
<223> n = T or U

<400> 312  
ngcagacnnc aangcnng

19

<210> 313  
<211> 19  
<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 1, 9, 13, 15, 19

<223> n = T or U

<400> 313

naagcaagnc acngnggcn

19

<210> 314

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 6, 10, 13, 16

<223> n = T or U

<400> 314

cngagncgan aanacnagc

19

<210> 315

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 3, 9, 10, 13

<223> n = T or U

<400> 315

acnagccann agnaaagag

19

<210> 316

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 15, 16, 18  
<223> n = T or U

<400> 316  
caacagcaga gaccnngnc

19

<210> 317  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 7, 11, 12  
<223> n = T or U

<400> 317  
anagcanacc nngaaccag

19

<210> 318  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 5, 7, 12, 17  
<223> n = T or U

<400> 318  
cancnngagg cnaagangg

19

<210> 319  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 4, 11, 16, 18  
<223> n = T or U

<400> 319  
agnnaccaga ngccancng 19

<210> 320  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 3, 5, 8, 10, 13, 16  
<223> n = T or U

<400> 320  
aancnacncn ganagnnga 19

<210> 321  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 2, 3, 4, 6, 17  
<223> n = T or U

<400> 321  
gnnncngaag ccaacanca 19

<210> 322  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 6, 7, 9, 14, 17  
<223> n = T or U

<400> 322  
ncaacnnanc accnccnga 19

<210> 323  
<211> 19  
<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 7, 12, 13, 15

<223> n = T or U

<400> 323

aagaacnaac anngnagag

19

<210> 324

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 13, 16

<223> n = T or U

<400> 324

gnagacaaca ggngcngca

19

<210> 325

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

<222> 2, 4, 7, 9, 11, 14, 15, 17

<223> n = T or U

<400> 325

angnccncng naannangg

19

<210> 326

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> based on Homo sapiens.  
Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature  
<222> 1, 4, 5, 9, 16  
<223> n = T or U

<400> 326  
nacnnggcna gaacangga

19

<210> 327  
<211> 19  
<212> DNA  
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<220>  
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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<221> misc\_feature

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<223> n = T or U

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<210> 346

<211> 19

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<210> 354

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<223> n = T or U

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<222> 10, 11, 16, 18, 19

<223> n = T or U

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<210> 358

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<223> n = T or U

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<223> n = T or U



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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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<210> 385

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Each nucleobase may be part of a ribonucleotide,  
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<223> n = T or U

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<210> 386

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<223> n = T or U

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<210> 387

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<223> n = T or U

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<223> n = T or U

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<223> n = T or U

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

<221> misc\_feature

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<223> n = T or U

<400> 453

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19

<210> 454

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<213> Artificial Sequence

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Each nucleobase may be part of a ribonucleotide,  
deoxyribonucleotide, or nucleotide analog

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19

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Each nucleobase may be part of a ribonucleotide,  
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Each nucleobase may be part of a ribonucleotide,  
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